



PATENT
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John B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Buchter-Larsen et al.
Filed : November 5, 1999
Serial No. : 09/423,126
For : A PROCESS FOR PREPARING AN ANTI-OXIDANT
Art Unit : 1638
Examiner : Russell Kallis

745 Fifth Avenue, New York, NY 10151

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Anne-Marie C. Yvon, Reg. No. 52,390

(Name of Applicant, Assignee or Registered Representative)

Anne-Marie C. Yvon
Signature

May 24, 2004

Date of Signature

COMMUNICATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Enclosed is a certified copy of priority documents for the above named application. Applicants hereby claim priority under 35 U.S.C. §§119 and 120 from International Patent Application No. PCT/IB98/00708 and United Kingdom Patent Application No. GB 9709161.5.

Acknowledgment of the claim of priority and of the receipt of said certified copy is respectfully requested.

Respectfully submitted,
FROMMER LAWRENCE & HAUG LLP

By: *Anne-Marie C. Yvon*
Thomas J. Kowalski, Esq.
Reg. No. 32,147

Anne-Marie C. Yvon, Ph.D.
Reg. No. 52,390
T:(212) 588-0800



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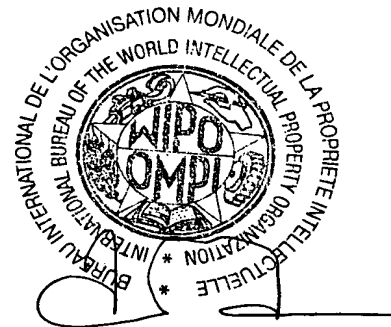
International Application No. } PCT/IB 98 / 00708 International Filing Date } 06 MAI 1998
Demande internationale n° } Date du dépôt international }
06.05.98

Geneva/Genève, **28 APRIL 2004**

(28.04.04)

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J.-L. Baron

**Head, PCT Receiving Office Section
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PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

PCT / IB 98 / 00708
International Application No.

06 MAY 1998 (06.05.98)
International Filing Date

INTERNATIONAL BUREAU OF WIPO
PCT International Application
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) PCT 500 1 CTH

Ro

Box No. I TITLE OF INVENTION A PROCESS OF PREPARING AN ANTI-OXIDANT	
Box No. II APPLICANT	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.) Danisco A/S Langebrogade 1 PO Box 17 DK-1001 Copenhagen K Denmark	
<input type="checkbox"/> This person is also inventor. Telephone No. Facsimile No. Teleprinter No.	
State (i.e. country) of nationality: Denmark	State (i.e. country) of residence: Denmark
This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input checked="" type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box	
Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.) BUCHTER-LARSEN, Akse1 Amager Felledves 27 DK-2300 Copenhagen Denmark	
This person is: <input type="checkbox"/> applicant only <input checked="" type="checkbox"/> applicant and inventor <input type="checkbox"/> inventor only (If this check-box is marked, do not fill in below.)	
State (i.e. country) of nationality: Denmark	State (i.e. country) of residence: Denmark
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<input checked="" type="checkbox"/> Further applicants and/or (further) inventors are indicated on a continuation sheet.	
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The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: <input checked="" type="checkbox"/> agent <input type="checkbox"/> common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) HARDING, Charles Thomas D Young & Co 21 New Fetter Lane London EC4A 1DA United Kingdom	
Telephone No. +44 1703 634816 Facsimile No. +44 1703 224262 Teleprinter No. 477667 YOUNGS G	
<input type="checkbox"/> Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

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MARCUSSEN, Ian
Knabrostrede 25
DK-1210 Copenhagen
Denmark

This person is:

- ☐ applicant only
☒ applicant and inventor
☐ inventor only (If this check-box is marked, do not fill in below.)

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This person is:

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This person is:

- ☐ applicant only
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State (i.e. country) of nationality:

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This person is applicant for the purposes of:

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Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

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The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

Use this box in the following cases:

1. If, in any of the Boxes, the space is insufficient to furnish all the information:

in particular:

- (i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available;
- (ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked;
- (iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America;
- (iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents;
- (v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part";
- (vi) if there are more than three earlier applications whose priority is claimed;

in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient;

in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below;

in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;

in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;

in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;

in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;

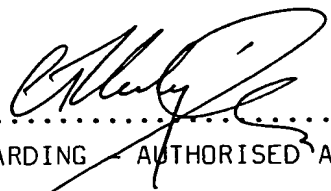
in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.

2. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty:

in such case, write "Statement Concerning Non-Prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below.

Continuation of Box No. IV

PURVIS, William Michael Cameron
COTTER, Ivan John
PILCH, Adam John Michael
CRISP, David Norman
ROBINSON, Nigel Alexander Julian
HARRIS, Ian Richard
HARDING, Charles Thomas
TURNER, James Arthur
PRICE, Paul Anthony King
PRATT, Richard Wilson
MALLALIEU, Catherine Louise
HOLMES, Miles Keeton
HORNER, David Richard
MASCHIO, Antonio
NACHSHEN, Neil Jacob
POTTER, Julian Mark

Box No. VI PRIORITY CLAIM		Further priority claims are indicated in the Supplemental Box <input type="checkbox"/>	
The priority of the following earlier application(s) is hereby claimed:			
Country (in which, or for which, the application was filed)	Filing Date (day/month/year)	Application No.	Office of filing (only for regional or international application)
item (1) United Kingdom	(06.05.97) ^A 06 May 1997	9709161.5	
item (2)			
item (3)			
Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):			
<input type="checkbox"/> The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): _____			
Box No. VII INTERNATIONAL SEARCHING AUTHORITY			
Choice of International Searching Authority (ISA) (If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used): ISA / <u>EP</u> ^A			
Earlier search Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request: Country (or regional Office): _____ Date (day/month/year): _____ Number: _____			
Box No. VIII CHECK LIST			
This international application contains the following number of sheets: 1. request : 5 sheets 2. description : 48 sheets 3. claims : 3 sheets 4. abstract : 1 sheets 5. drawings : _____ sheets Total : 57 sheets		This international application is accompanied by the item(s) marked below: 1. <input type="checkbox"/> separate signed power of attorney 2. <input type="checkbox"/> copy of general power of attorney 3. <input type="checkbox"/> statement explaining lack of signature 4. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s): 5. <input type="checkbox"/> fee calculation sheet 6. <input type="checkbox"/> separate indications concerning deposited microorganisms 7. <input type="checkbox"/> nucleotide and/or amino acid sequence listing (diskette) 8. <input checked="" type="checkbox"/> other (specify): Letter	
Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.			
Box No. IX SIGNATURE OF APPLICANT OR AGENT			
Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).			
 C T HARDING - AUTHORISED AGENT			

For receiving Office use only		2. Drawings: <input type="checkbox"/> received: <input type="checkbox"/> not received:
1. Date of actual receipt of the purported international application:	06 MAY 1998 06 05 98	
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:		
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A PROCESS OF PREPARING AN ANTI-OXIDANT

The present invention relates to a process of preparing an anti-oxidant.

5 An anti-oxidant prevents, inhibits or reduces the oxidation rate of an oxidisable medium. In particular, anti-oxidants are used for the preservation of food, especially when the food is or comprises a fat. Typical chemical anti-oxidants include aromatic amines, substituted phenols and sulphur compounds. Examples of anti-oxidants for food products are polyvinylpolypyrrolidone, dithiothreitol, sulphur dioxide, synthetic
10 γ -tocopherol, δ -tocopherol, L-ascorbic acid, sodium L-ascorbate, calcium L-ascorbate, ascorbyl palmitate, propyl gallate, octyl gallate, dodecyl gallate, lecithin, diphenylamine ethoxyquin and butylated hydroxytoluene. Two commonly used anti-oxidants are GRINDOX 142 (obtained from Danisco A/S) and GRINDOX 1029 (obtained from Danisco A/S).

15

Typically, anti-oxidants are added to foodstuffs, such as beverages.

For example, anti-oxidants are used in the preparation of alcoholic beverages such as beer, cider, ale etc.. In particular, there is a wide spread use of anti-oxidants in the
20 preparation of wine. In this regard, Butzke and Bisson in Agro-Food-Industry Hi-Tech (July/August 1996 pages 26-30) present a review of wine manufacture.

According to Butzke and Bisson (*ibid*):

25 *"Wine is the product of the natural fermentation of grape must or juice. In the case of red wine, the skins are present during the initial fermentation to allow extraction of pigment and important flavour and aroma constituents from the skin. The term "must" refers to the crushed whole grapes. In the case of white wine production, skins are*
30 *removed prior to fermentation and only the juice is retained and processed.*

5 *Grapes are harvested and brought directly to the winery from the field. The grapes are then crushed at the winery and the must either transferred to a tank for fermentation (red wine) or pressed to separate juice from the skin and seeds (white wine). In this latter case, the*
10 *juice is then transferred to a tank for fermentation. The tanks may either be inoculated with a commercial wine strain of Saccharomyces or allowed to undergo a natural or uninoculated fermentation. In a natural fermentation, Saccharomyces cells are greatly outnumbered by wild (non-Saccharomyces) yeast and bacteria at the beginning of fermentation. By the end of the fermentation Saccharomyces is the dominant and most often only organism isolateable. Inoculation with a commercial wine strain or with fermenting juice or must changes the initial ratio of the numbers of different microorganisms, allowing Saccharomyces to dominate the fermentation much earlier.*

15 *The metabolic activity of microorganisms in wine results in the production of aroma and flavour compounds some of which are highly objectionable to the consumer and all of which are distinct from the compounds responsible for the varietal character of the wine.*
20 *Sulphur dioxide addition prevents chemical oxidation reactions and in this sense is an important stabilizer of the natural grape aroma and flavour. It may be added to the must or juice to preserve flavour, not necessarily as an antimicrobial agent. However, its antimicrobial activity must be considered when choosing a strain to be genetically*
25 *modified for wine production."*

Hence, potentially harmful chemicals - such as sulphur dioxide - are used in wine manufacture.

30 The present invention seeks to overcome any problems associated with the prior art methods of preparing foodstuffs with antioxidants.

According to a first aspect of the present invention there is provided a process of preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; and wherein the anti-oxidant is prepared from a glucan by use of recombinant DNA techniques.

5

According to a second aspect of the present invention there is provided a process of preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; and wherein the anti-oxidant is prepared by use of a recombinant glucan lyase.

10

According to a third aspect of the present invention there is provided a medium prepared by the process according to the present invention.

Other aspects of the present invention include:

15

Use of anhydrofructose as an anti-oxidant for a medium comprising at least one other component, wherein the anhydrofructose is prepared *in situ* in the medium.

20

Use of anhydrofructose as a means for imparting or improving stress tolerance in a plant, wherein the anhydrofructose is prepared *in situ* in the plant.

25

Use of anhydrofructose as a means for imparting or improving the transformation of a grape, wherein the anhydrofructose is prepared *in situ* in the grape.

30

Use of anhydrofructose as a means for increasing antioxidant levels in a foodstuff (preferably a fruit or vegetable, more preferably a fresh fruit or a fresh vegetable), wherein the anhydrofructose is prepared *in situ* in the foodstuff.

Use of anhydrofructose as a pharmaceutical in a foodstuff, wherein the anhydrofructose is prepared *in situ* in the foodstuff.

5 A method of administering a foodstuff comprising anhydrofructose, wherein the anhydrofructose is in a pharmaceutically acceptable amount and acts as a pharmaceutical; and wherein the anhydrofructose has been prepared *in situ* in the foodstuff.

10 Use of anhydrofructose as a nutraceutical in a foodstuff, wherein the anhydrofructose is prepared *in situ* in the foodstuff.

15 A method of administering a foodstuff comprising anhydrofructose, wherein the anhydrofructose is in a nutraceutically acceptable amount and acts as a nutraceutical; and wherein the anhydrofructose has been prepared *in situ* in the foodstuff.

Use of glucan lyase as a means for imparting or improving stress tolerance in a plant, wherein the glucan lyase is prepared *in situ* in the plant.

20 Use of glucan lyase as a means for imparting or improving the transformation of a grape, wherein the glucan lyase is prepared *in situ* in the grape.

25 Use of glucan lyase as a means for increasing antioxidant levels in a foodstuff (preferably a fruit or vegetable, more preferably a fresh fruit or a fresh vegetable), wherein the glucan lyase is prepared *in situ* in the foodstuff.

Use of glucan lyase in the preparation of a pharmaceutical in a foodstuff, wherein the glucan lyase is prepared *in situ* in the foodstuff.

30 A method of administering a foodstuff comprising an antioxidant, wherein the antioxidant is in a pharmaceutically acceptable amount and acts as a pharmaceutical; and wherein the antioxidant has been prepared *in situ* in the

foodstuff from a glucan lyase.

Use of glucan lyase in the preparation of a nutraceutical in a foodstuff, wherein the glucan lyase is prepared *in situ* in the foodstuff.

5

A method of administering a foodstuff comprising an antioxidant, wherein the antioxidant is in a nutraceutically acceptable amount and acts as a nutraceutical; and wherein the antioxidant has been prepared *in situ* in the foodstuff from a glucan lyase.

10

Use of a nucleotide sequence coding for a glucan lyase as a means for imparting or improving stress tolerance in a plant, wherein the nucleotide sequence is expressed *in situ* in the plant.

15

Use of a nucleotide sequence coding for a glucan lyase as a means for imparting or improving the transformation of a grape, wherein the nucleotide sequence is expressed *in situ* in the grape.

20

Use of a nucleotide sequence coding for a glucan lyase as a means for increasing antioxidant levels in a foodstuff (preferably a fruit or vegetable, more preferably a fresh fruit or a fresh vegetable), wherein the nucleotide sequence is expressed *in situ* in the foodstuff.

25

Use of a nucleotide sequence coding for a glucan lyase as a means for creating a pharmaceutical in a foodstuff, wherein the nucleotide sequence is expressed *in situ* in the foodstuff.

30

A method of administering a foodstuff comprising an antioxidant, wherein the antioxidant is in a pharmaceutically acceptable amount and acts as a pharmaceutical; and wherein the antioxidant has been prepared *in situ* in the foodstuff by means of a nucleotide sequence coding for a glucan lyase.

Use of a nucleotide sequence coding for a glucan lyase as a means for creating a nutraceutical in a foodstuff, wherein the nucleotide sequence is expressed *in situ* in the foodstuff.

5 A method of administering a foodstuff comprising an antioxidant, wherein the antioxidant is in a nutraceutically acceptable amount and acts as a nutraceutical; and wherein the antioxidant has been prepared *in situ* in the foodstuff by means of a nucleotide sequence coding for a glucan lyase.

10 The term "nutraceutical" means a compound that is capable of acting as a nutrient (i.e. it is suitable for, for example, oral administration) as well as being capable of exhibiting a pharmaceutical effect and/or cosmetic effect.

 In contrast to the usual practice of adding anti-oxidants media, such as foodstuffs, we
15 have now found that particular anti-oxidants can be prepared *in situ* in the medium.

 The *in situ* preparation of anti-oxidants is particularly advantageous in that less, or even no, additional anti-oxidants need be added to the medium, such as a food product.

20 The present invention is also believed to be advantageous as it provides a means of improving stress tolerance of plants.

 The present invention is also advantageous as it provides a means for viably
25 transforming grape.

 The present invention is further advantageous in that it enables the levels of antioxidants in foodstuffs to be elevated. This may have beneficial health implications. In this regard, recent reports (e.g. Biotechnology Newswatch April 21
30 1997 "*Potent Antioxidants, as strong as those in fruit, found in coffee*" by Marjorie Shaffer) suggest that antioxidants have a pharmaceutical benefit, for example in preventing or suppressing cancer formation.

General *in situ* preparation of antioxidants in plants has been previously reviewed by Badiani *et al* in Agro-Food-Industry Hi-Tech (March/April 1996 pages 21-26). It is to be noted, however, that this review does not mention preparing *in situ* antioxidants from a glucan, let alone by use of a recombinant glucan lyase.

5

Preferably, the glucan comprises α -1,4 links.

Preferably, the glucan is starch or a unit of starch.

- 10 Preferably, the glucan is a substrate for a recombinant enzyme such that contact of the glucan with the recombinant enzyme yields the anti-oxidant.

Preferably, the enzyme is a glucan lyase.

- 15 Preferably, the enzyme is an α -1,4-glucan lyase.

Preferably, the enzyme comprises any one of the sequences shown as SEQ ID Nos 1-6, or a variant, homologue or fragment thereof.

- 20 Preferably, the enzyme is any one of the sequences shown as SEQ ID Nos 1-6.

Preferably, the enzyme is encoded by a nucleotide sequence comprising any one of the sequences shown as SEQ ID Nos 7-12, or a variant, homologue or fragment thereof.

25

Preferably, the enzyme is encoded by a nucleotide sequence having any one of the sequences shown as SEQ ID Nos 7-12.

Preferably, the anti-oxidant is anhydrofructose.

30

Preferably, the anti-oxidant is 1,5-D-anhydrofructose.

Preferably, the medium is, or is used in the preparation of, a foodstuff.

Preferably, the foodstuff is a beverage.

5 Preferably, the beverage is an alcoholic beverage.

Preferably, the beverage is a wine.

10 Preferably, the anti-oxidant is prepared *in situ* in the component and is then released into the medium.

Preferably, the component is a plant or a part thereof.

15 Preferably, the component is all or part of a cereal or a fruit.

Preferably, the component is all or part of a grape.

20 The medium may be used as or in the preparation of a foodstuff, which includes beverages. In the alternative, the medium may be for use in polymer chemistry. In this regard, the *in situ* generated anti-oxidants could therefore act as oxygen scavengers during, for example, the synthesis of polymers, such as the synthesis of bio-degradable plastic.

25 In accordance with the present invention, the anti-oxidant (preferably anhydrofructose) is prepared *in situ* in the medium. In other words, the antioxidant (preferably anhydrofructose) that is prepared *in situ* in the medium is used as an anti-oxidant in the medium. In one embodiment, the antioxidant (preferably anhydrofructose) that is prepared *in situ* in the medium is used as the main anti-oxidant in the medium.

30 The term "*in situ* in the medium" as used herein includes the anti-oxidant being prepared by action of a recombinant enzyme expressed by the component on a glucan - which glucan is a substrate for the enzyme. The term also includes the anti-oxidant

being prepared by action of a recombinant enzyme expressed by the component on a glucan - which glucan is a substrate for the enzyme - within the component and the subsequent generation of the anti-oxidant. The term also includes the recombinant enzyme being expressed by the component and then being released into the medium, which enzyme acts on a glucan - which glucan is a substrate for the enzyme - present in the medium to form the anti-oxidant in the medium. The term also covers the presence or addition of another component to the medium, which component then expresses a recombinant nucleotide sequence which results in exposure of part or all of the medium to an anti-oxidant, which anti-oxidant may be a recombinant enzyme or a recombinant protein expressed and released by the other component, or it may be a product of a glucan - which glucan is a substrate for the enzyme - within the medium that has been exposed to the recombinant enzyme or the recombinant protein.

The term "by use of recombinant DNA techniques" as used herein includes the anti-oxidant being any obtained by use of a recombinant enzyme or a recombinant protein, which enzyme or protein acts on the glucan. The term also includes the anti-oxidant being any obtained by use of an enzyme or protein, which enzyme or protein acts on a recombinant glucan.

The term "starch" in relation to the present invention includes native starch, degraded starch, modified starch, including its components amylose and amylopectin, and the glucose units thereof.

The terms "variant", "homologue" or "fragment" in relation to the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant amino acid sequence has α -glucan lyase activity, preferably having at least the same activity of any one of the enzymes shown as SEQ ID No. 1-6. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant enzyme has α -glucan lyase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to any one of the sequences shown as SEQ ID No.s 1-6. More

preferably there is at least 95%, more preferably at least 98%, homology to any one of the sequences shown as SEQ ID No. 1-6.

5 The terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence coding for the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for an enzyme having α -glucan lyase activity, preferably having at least the same activity of any one of the enzymes shown as SEQ ID No. 1-6. In particular, the term "homologue" covers
10 homology with respect to structure and/or function providing the resultant nucleotide sequence codes for an enzyme having α -glucan lyase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to any one of the sequences shown as SEQ ID No. 7-12. More preferably there is at least 95%, more preferably at least 98%,
15 homology to any one of the sequences shown as SEQ ID No. 7-12.

The above terms are synonymous with allelic variations of the sequences.

20 The present invention also covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention.

The term "nucleotide" in relation to the present invention includes cDNA.

25 According to the present invention there is therefore provided a method of preparing *in situ* in an oxidisable medium an anti-oxidant. In a preferred embodiment, the anti-oxidant is anhydrofructose, more preferably 1,5-D-anhydrofructose. 1,5-D-anhydrofructose has been chemically synthesised (Lichtenthaler in Tetrahedron Letters Vol 21 pp 1429-1432). 1,5-D-anhydrofructose is further discussed in WO 95/10616, WO 95/10618 and GB-B-2294048.

The main advantages of using 1,5-D-anhydrofructose as an anti-oxidant are that it is a natural product, it is non-metabolisable, it is easy to manufacture, it is water-soluble, and it is generally non-toxic.

- 5 According to WO 95/10616, WO 95/10618 and GB-B-2294048, 1,5-D-anhydrofructose may be prepared by the enzymatic modification of substrates based on α -1,4-glucan by use of the enzyme α -1,4-glucan lyase. A typical α -1,4-glucan based substrate is starch.
- 10 Today, starches have found wide uses in industry mainly because they are cheap raw materials. There are many references in the art to starch. For example, starch is discussed by Salisbury and Ross in Plant Physiology (Fourth Edition, 1991, Published by Wadsworth Publishing Company - especially section 11.7). In short, however, starch is one of the principal energy reserves of plants. It is often found in colourless
- 15 plastids (amyloplasts), in storage tissue and in the stroma of chloroplasts in many plants. Starch is a polysaccharide carbohydrate. It comprises two main components: amylose and/or amylopectin. Both amylose and/or amylopectin consist of straight chains of α (1,4)-linked glucose units (ie glycosyl residues) but in addition amylopectin includes α (1,6) branched glucose units.
- 20 Some of the glucan lyases discussed in WO 95/10616 and WO 95/10618 that are suitable for producing 1,5-D-anhydrofructose from starch are shown as SEQ I.D. No.s 1-4. Some of the glucan lyases discussed in GB-B-2294048 that are suitable for producing 1,5-D-anhydrofructose from starch are shown as SEQ I.D. No.s 5-6.
- 25 Some of the nucleotide sequences coding for glucan lyases discussed in WO 95/10616 and WO 95/10618 that are suitable for producing 1,5-D-anhydrofructose from starch are shown as SEQ I.D. No.s 7-10. Some of the nucleotide sequences coding for glucan lyases discussed in GB-B-2294048 that are suitable for producing 1,5-D-
- 30 anhydrofructose from starch are shown as SEQ I.D. No.s 11-12.

A further glucan lyase is discussed in WO 94/09122.

The recombinant nucleotide sequences coding for the enzyme may be cloned from sources such as a fungus; preferably *Morchella costata* or *Morchella vulgaris*, or from a fungally infected algae, preferably *Gracilariopsis lemaneiformis*, or from algae lone, preferably *Gracilariopsis lemaneiformis*.

5

In a preferred embodiment, the 1,5-D-anhydrofructose is prepared *in situ* by treating an α -1,4-glucan with a recombinant α -1,4-glucan lyase, such as any one of those presented as SEQ I.D. No.s 1-6.

10 Detailed commentary on how to prepare the enzymes shown as sequences SEQ I.D. No.s 1-6 may be found in the teachings of WO 95/10616, WO 95/10618 and GB-B-2294048. Likewise, detailed commentary on how to isolate and clone the nucleotide sequences SEQ I.D. No.s 7-12 may be found in the teachings of WO 95/10616, WO 95/10618 and GB-B-2294048.

15

If the glucan contains links other than and in addition to the α -1,4- links the recombinant α -1,4-glucan lyase can be used in conjunction with a suitable reagent that can break the other links - such as a recombinant hydrolase - preferably a recombinant glucanohydrolase.

20

General teachings of recombinant DNA techniques may be found in Sambrook, J., Fritsch, E.F., Maniatis T. (Editors) Molecular Cloning. A laboratory manual. Second edition. Cold Spring Harbour Laboratory Press. New York 1989.

25 In order to express a nucleotide sequence, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a
30 prokaryotic host is used then the gene may need to be suitably modified before transformation - such as by removal of introns.

In one embodiment, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*. A transgenic *Aspergillus* can be prepared by following the teachings of Rambosek, J. and Leach, J. 1987 (Recombinant DNA in filamentous fungi: Progress and Prospects. CRC Crit. Rev. Biotechnol. 6:357-393), Davis R.W. 5 1994 (Heterologous gene expression and protein secretion in *Aspergillus*. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus: 50 years on*. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp 525-560), Ballance, D.J. 1991 (Transformation systems for Filamentous Fungi and an Overview of Fungal Gene structure. In: Leong, S.A., Berka R.M. (Editors) Molecular Industrial 10 Mycology. Systems and Applications for Filamentous Fungi. Marcel Dekker Inc. New York 1991. pp 1-29) and Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus: 50 years on*. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp. 641-666). However, the following commentary provides a summary of those teachings for producing 15 transgenic *Aspergillus*.

For almost a century, filamentous fungi have been widely used in many types of industry for the production of organic compounds and enzymes. For example, traditional japanese koji and soy fermentations have used *Aspergillus sp.* Also, in this 20 century *Aspergillus niger* has been used for production of organic acids particular citric acid and for production of various enzymes for use in industry.

There are two major reasons why filamentous fungi have been so widely used in industry. First filamentous fungi can produce high amounts of extracellular products, 25 for example enzymes and organic compounds such as antibiotics or organic acids. Second filamentous fungi can grow on low cost substrates such as grains, bran, beet pulp etc. The same reasons have made filamentous fungi attractive organisms as hosts for heterologous expression of recombinant enzymes according to the present invention.

In order to prepare the transgenic *Aspergillus*, expression constructs are prepared by inserting a requisite nucleotide sequence into a construct designed for expression in filamentous fungi.

- 5 Several types of constructs used for heterologous expression have been developed. These constructs can contain a promoter which is active in fungi. Examples of promoters include a fungal promoter for a highly expressed extracellular enzyme, such as the glucoamylase promoter or the α -amylase promoter. The nucleotide sequence can be fused to a signal sequence which directs the protein encoded by the nucleotide
10 sequence to be secreted. Usually a signal sequence of fungal origin is used. A terminator active in fungi ends the expression system.

- Another type of expression system has been developed in fungi where the nucleotide sequence can be fused to a smaller or a larger part of a fungal gene encoding a stable
15 protein. This can stabilize the protein encoded by the nucleotide sequence. In such a system a cleavage site, recognized by a specific protease, can be introduced between the fungal protein and the protein encoded by the nucleotide sequence, so the produced fusion protein can be cleaved at this position by the specific protease thus liberating the protein encoded by the nucleotide sequence. By way of example, one
20 can introduce a site which is recognized by a KEX-2 like peptidase found in at least some *Aspergilli*. Such a fusion leads to cleavage *in vivo* resulting in protection of the expressed product and not a larger fusion protein.

- Heterologous expression in *Aspergillus* has been reported for several genes coding for
25 bacterial, fungal, vertebrate and plant proteins. The proteins can be deposited intracellularly if the nucleotide sequence is not fused to a signal sequence. Such proteins will accumulate in the cytoplasm and will usually not be glycosylated which can be an advantage for some bacterial proteins. If the nucleotide sequence is equipped with a signal sequence the protein will accumulate extracellularly.

30

With regard to product stability and host strain modifications, some heterologous proteins are not very stable when they are secreted into the culture fluid of fungi.

Most fungi produce several extracellular proteases which degrade heterologous proteins. To avoid this problem special fungal strains with reduced protease production have been used as host for heterologous production.

5 For the transformation of filamentous fungi, several transformation protocols have been developed for many filamentous fungi (Ballance 1991, *ibid*). Many of them are based on preparation of protoplasts and introduction of DNA into the protoplasts using PEG and Ca^{2+} ions. The transformed protoplasts then regenerate and the transformed fungi are selected using various selective markers. Among the markers
10 used for transformation are a number of auxotrophic markers such as *argB*, *trpC*, *niaD* and *pyrG*, antibiotic resistance markers such as benomyl resistance, hygromycin resistance and phleomycin resistance. A commonly used transformation marker is the *amdS* gene of *A. nidulans* which in high copy number allows the fungus to grow with acrylamide as the sole nitrogen source.

15

In another embodiment the transgenic organism can be a yeast. In this regard, yeast have also been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in
20 *Saccharomyces cerevisiae* has been reviewed by Goodey *et al* (1987, Yeast Biotechnology, D R Berry *et al*, eds, pp 401-429, Allen and Unwin, London) and by King *et al* (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

25 For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the
30 organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

5

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

- 10 In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a
15 signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

- For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* can be prepared by following the teachings
20 of Hinnen *et al* (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

- The transformed yeast cells are selected using various selective markers. Among the
25 markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

- Another host organism is a plant. In this regard, the art is replete with references for
30 preparing transgenic plants. Two documents that provide some background commentary on the types of techniques that may be employed to prepare transgenic plants are EP-B-0470145 and CA-A-2006454 - some of which commentary is

presented below.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries a recombinant nucleotide sequence and which is capable of introducing the nucleotide sequence into the genome of an organism, such as a plant, and wherein the nucleotide sequence is capable of preparing *in situ* an anti-oxidant.

The vector system may comprise one vector, but it can comprise at least two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* (An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208).

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

The nucleotide sequence of the present invention should preferably be inserted into

the Ti-plasmid between the border sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

5

As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic
10 construct. Preferably, the vector system is an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

15 In the construction of a transgenic plant the nucleotide sequence or construct or vector of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli.*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined
20 above has been constructed in *E. coli.* it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the first nucleotide sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the promoter or nucleotide sequence or construct
25 of the invention, which DNA is subsequently transferred into the plant cell to be modified.

As reported in CA-A-2006454, a large number of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the
30 transformed cells. The vectors contain for example pBR322, the pUC series, the M13 mp series, pACYC 184 etc. In this way, the promoter or nucleotide or construct of the present invention can be introduced into a suitable restriction position

in the vector. The contained plasmid is used for the transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered and then analysed - such as by any one or more of the following techniques: sequence analysis, restriction analysis, electrophoresis and
5 further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted or selectively amplified by PCR techniques and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

10 After each introduction method of the nucleotide sequence or construct or vector according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as
15 flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasterdam, 1985, Chapter V; Fraley, *et al.*, Crit. Rev. Plant Sci., 4:1-46; and An *et al.*, EMBO J. (1985) 4:277-284.

20 Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. *et al.* (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant
25 Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant.

30 Typically, with direct infection of plant tissues by *Agrobacterium* carrying the first nucleotide sequence or the construct, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the

plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The inoculated plant or plant part is then grown on a suitable culture medium.

When plant cells are constructed, these cells are grown and, optionally, maintained
5 in a medium according to the present invention following well-known tissue culturing methods - such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc, but wherein the culture medium comprises a component according to the present invention. Regeneration of the transformed cells into genetically modified plants may
10 be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting the transformed shoots and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Further teachings on plant transformation may be found in EP-A-0449375.
15

Reference may even be made to Spngstad *et al* (1995 Plant Cell Tissue Organ Culture
40 pp 1-15) as these authors present a general overview on transgenic plant construction.

20 In one embodiment, the plant is a grapevine. There are a number of teachings in the art on how to prepare transformed grapevines. For example, reference may be made to Baribault *et al* (J Exp Bot 41 (229) 1990 1045-1050), Baribault *et al* (Plant Cell Rep 8 (3) 1989 137-140), Scorza *et al* (J Am Soc Horticultural Science 121 (4) 1996 616-619), Kikkert *et al* (Plant Cell Reports 15 (5) 1996 311-316), Golles *et al* (Acta
25 Hortic 1997 vol 447 Number: Horticultural Biotechnology in Vitro Culture and Breeding Pages 265-275), Gray and Scorza (WO-A-97/49277) and Simon Robinson *et al* (Conference abstracts and paper presented in Biotechnology - Food and Health for the 21st Century, Adelaide, Australia, 1998). By way of example Robinson *et al* (*ibid*) disclose a method for transforming grapevine wherein somatic embryos are
30 induced on callus formed from another tissue and *Agrobacterium* infection is used to transfer target genes into the embryo tissue.

Further reference may be made to the teachings of Andrew Walker in Nature Biotechnology (Vol 14, May 1996, page 582) who states that:

5 *"The grape, one of the most important fruit plants in the world, has been difficult to engineer because of its high levels of tannins and phenols, which interfere with cell culture and transformation; the compounds oxidize quickly and promote the decay of grape cells."*

10 In that same edition of Nature Biotechnology, Perl *et al* (pages 624-628) report on the use of the combination of polyvinylpyrrolidone and dithiothreitol to improve the viability of grape transformation during *Agrobacterium* infection.

15 Hence, the present invention provides an alternative means for transforming grape. In this regard, the antioxidant that is prepared *in situ* by a grape transformed in accordance with the present invention improves the viability of grape transformation during *Agrobacterium* infection.

20 Thus, according to one aspect of the present invention, there is provided the use of an antioxidant prepared *in situ* in order to effectively transform a grape.

25 In some instances, it is desirable for the recombinant enzyme or protein to be easily secreted into the medium to act as or to generate an anti-oxidant therein. In such cases, the DNA encoding the recombinant enzyme is fused to *inter alia* an appropriate signal sequence, an appropriate promoter and an appropriate terminator from the chosen host.

30 For example, for expression in *Aspergillus niger* the *gpdA* (from the Glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*) promoter and signal sequence is fused to the 5' end of the DNA encoding the mature lyase. The terminator sequence from the *A. niger* *trpC* gene is placed 3' to the gene (Punt, P.J. et al 1991 - (1991): J. Biotech. 17, 19-34). This construction is inserted into a vector containing a replication origin and selection origin for *E. coli* and a selection marker

for *A. niger*. Examples of selection markers for *A. niger* are the *amdS* gene, the *argB* gene, the *pyrG* gene, the *hygB* gene, the *BmlR* gene which all have been used for selection of transformants. This plasmid can be transformed into *A. niger* and the mature lyase can be recovered from the culture medium of the transformants.

5 Eventually the construction could be transformed into a protease deficient strain to reduce the proteolytic degradation of the lyase in the medium (Archer D.B. et al 1992 -Biotechnol. Lett. 14, 357-362).

10 In addition, and as indicated above, aside from using *Aspergillus niger* as the host, there are other industrial important microorganisms which could be used as expression systems. Examples of these other hosts include: *Aspergillus oryzae*, *Aspergillus sp.*, *Trichoderma sp.*, *Saccharomyces cerevisiae*, *Kluyveromyces sp.*, *Hansenula sp.*, *Pichia sp.*, *Bacillus subtilis*, *B. amyloliquefaciens*, *Bacillus sp.*, *Streptomyces sp.* or *E. coli*.

15 In accordance with the present invention, a suitable marker or selection means may be introduced into the host that is to be transformed with the nucleotide sequence. Examples of suitable markers or selection means are described in any one of WO-A-93/05163, WO-A-94/20627, GB patent application No. 9702591.0 (filed 7 February 1997), GB patent application No. 9702576.1 (filed 7 February 1997), GB patent application No. 9702539.9 (filed 7 February 1997), GB patent application No. 9702510.0 (filed 7 February 1997) and GB patent application No. 9702592.8 (filed 7 February 1997).

25 In summation, the present invention relates to a process comprising preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; and wherein the anti-oxidant is prepared from a glucan by use of recombinant DNA techniques and/or the anti-oxidant is prepared by use of a recombinant glucan lyase.

In a preferred embodiment, the present invention relates to a process a process of preparing a medium that comprises an anti-oxidant and at least one other component. the process comprising preparing *in situ* in the medium the anti-oxidant; and wherein the anti-oxidant is prepared from a glucan by use of a recombinant glucan lyase.

5

In a more preferred embodiment, the present invention relates to a process of preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; wherein the anti-oxidant is prepared from a glucan by use of a recombinant glucan lyase; and
10 wherein the anti-oxidant is anhydro-fructose.

The present invention will now be described only by way of example.

TRANSGENIC GRAPE

15

Transformed grapes are prepared following the teachings of Perl *et al* (*ibid*) but wherein the use of the combination of polyvinylpolypyrrolidone and dithiothreitol is optional. In these studies, the grapes are transformed with any one of the nucleotide sequences presented as SEQ ID No. 7-12. The transformation leads to *in situ*
20 preparation of 1,5-D-anhydrofructose. The transformed grapes are beneficial for one or more of the reasons mentioned earlier.

Details on these studies are as follows.

25 Tissue-culture systems for transformation studies

The long term somatic embryogenic callus culture is developed from the vegetative tissues of anthers of *Vitis vinifera* CV Superior Seedless. Methods for another culture, induction of somatic embryogenesis and maintenance of embryogenic
30 cultures, are previously described (Perl *et al*, 1995, Plant Sci **104**: 193-200). Briefly, embryogenic calli are maintained on solidified (0.25% gelrite) MS medium (Murashige and Skoog, 1962, Physiol Plant **15**: 473-497) supplemented with 6%

sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 5 mg/L Indole-3-aspartic acid (IASP), 0.2 mg/L 6-benzyladenine (BAP) and 1 mg/L abscisic acid (ABA). Proembryogenic calli are induced by transferring the calli to MS medium supplemented with the same phytohormones, but 2,4-D is substituted with 2 mg/L 2-naphthoxyacetic acid (NOA). This stage is used for transformation experiments.

***Agrobacterium* strains**

For studying the sensitivity of grape embryogenic calli to the presence of different *Agrobacterium* strains, or for stable transformation experiments, cocultivation is attempted using the following *A. tumefaciens* strains: EHA 101-p492 (Perl *et al*, 1993, Bio/Technology **11**:715-718); LBA 4404-pGPTV (Becker *et al*, 1992, Plant Mol Biol **20**: 1195-1197); and GVE 3101-pPCV91 (Vancanneyt *et al*, 1990, Mol Gen Genet **220**: 245-250). These strains contain the binary vectors conferring resistance to kanamycin (*nptII*), basta (*bar*) and hygromycin (*hpt*), respectively, all under the control of the nopaline-synthase (NOS) promoter and terminator. Bacteria are cultured with the proper antibiotics in liquid LB medium for 24 hours at 28°C at 200 rpm.

Cocultivation

For studying the sensitivity of grape embryogenic calli to different *Agrobacterium* strains, bacterial cultures with different optical densities (0.1-0.7 at 630 nm) are prepared from an overnight culture of *Agrobacterium* strains. Bacteria are centrifuged 5 minutes, 5000 rpm and resuspended in antibiotic free McCown's Woody Plant Medium (WPM) (Lloyd and McCown, 1981, Int Plant Prop Soc Proc **30**: 421-427). Three grams fresh weight of embryogenic calli (7 days after transfer to NOA containing medium) are resuspended in 10 ml of overnight cultured bacterial suspensions for 5 minutes, dry blotted and transferred to Petri dishes containing regeneration medium [basal WPM medium supplemented with thidiazuron (TDZ) (0.5 mg/L), Zeatin riboside (ZR) (0.5 mg/L), and sucrose (3%)]. The regeneration medium is solidified with gelrite (0.25% w/v) and the calli, after initial drainage of excess bacteria, are cocultivated in the dark at 25°C for different times (5 minutes

up to 7 days). For stable transformation experiments, inoculum (OD 0.6 at 630 nm) is prepared from an overnight culture of LBA 4404 or GVE 3101. Bacteria are centrifuged 5 minutes, 5000 rpm and resuspended in antibiotic-free WPM medium. Embryogenic calli (3g fresh weight) are resuspended in 10 ml of bacteria for 5 minutes, dry blotted and transferred to Petri dishes containing solidified (0.25% w/v) gelrite regeneration medium supplemented with different antioxidants. The calli are cocultivated for 48 hours in the dark at 25°C.

Selective culture

10

Following 48 hours of cocultivation, the embryogenic callus is maintained in the dark for 7 days on antioxidant containing regeneration medium. Subsequently, the calli are collected on a sterile metal screen and transferred to fresh WPM regeneration medium at 25°C under 40 $\mu\text{E}/\text{m}^2/\text{s}$ (white fluorescent tubes). All regeneration media are supplemented with 400 mg/L claforan, 1.5 g/L malt extract and different selectable markers: kanamycin (50-500 mg/L), hygromycin (15 mg/L) and Basta (1-10 mg/L). Periodic increases in hygromycin concentration are used. The putative transformed calli are cultured on regeneration medium supplemented with 15 mg/L hygromycin. Every two weeks the regenerating calli are transferred to fresh medium supplemented with 20 and 25 mg/L hygromycin respectively. Control, untransformed grape calli are also cultured on selective media and are periodically exposed to increasing hygromycin concentrations. Green adventitious embryos, which developed on calli cultured for 8-10 weeks on selective regeneration medium, are transferred to germination medium. Embryo germination, rooting and subsequent plantlet development are induced on WPM as described (Perl *et al*, 1995, Plant Sci **104**: 193-200), supplemented with 25 mg/L hygromycin or 10 mg/L basta. Conversion of vitrified abnormal plantlets into normal-looking grape plantlets are obtained using solidified WPM medium supplemented with 0.1 mg/L NAA as described (Perl *et al*, 1995, Plant Sci **104**: 193-200).

30

TRANSGENIC POTATOES

General teachings on potato transformation may be found in our copending patent applications PCT/EP96/03053, PCT/EP96/03052 and PCT/EP94/01082 (the contents
5 of each of which are incorporated herein by reference).

For the present studies, the following protocol is adopted.

Plasmid construction

10

The disarmed *Agrobacterium tumefaciens* strain LBA 4404, containing the helper *vir* plasmid pRAL4404 (Hoekema *et al*, 1983 Nature **303** pp 179-180), is cultured on YMB agar ($K_2HPO_4 \cdot 3H_2O$ 660 mg l⁻¹, $MgSO_4$ 200 mg l⁻¹, NaCl 100 mg l⁻¹, mannitol 10 g l⁻¹, yeast extract 400 mg l⁻¹, 0.8% w/v agar, pH 7.0) containing 100 mg l⁻¹
15 rifampicin and 500 mg l⁻¹ streptomycin sulphate. Transformation with pVICTOR IV GNG E35S *nagB* IV2' or pVICTOR IV GNG *rbc nagB* IV2' or pVICTOR IV GNG E35S *nagB*' (which correspond to each of pVICTOR IV GNG E35S *nagB* IV2 or pVICTOR IV GNG *rbc nagB* IV2 or pVICTOR IV GNG E35S *nagB* but wherein each of those plasmids also contains any one of the nucleotide sequences shown as
20 SEQ ID No.s. 7-12 operatively linked to a functional promoter) is accomplished using the freeze-thaw method of Holters *et al* (1978 Mol Gen Genet **163** 181-187) and transformants are selected on YMB agar containing 100 mg l⁻¹ rifampicin and 500 mg l⁻¹ streptomycin, and 50 mg l⁻¹ gentamycin sulphate.

25 Transformation of plants

Shoot cultures of *Solanum tuberosum* cv Saturna are maintained on LS agar containing Murashige Skoog basal salts (Sigma M6899) (Murashige and Skoog, 1965, Physiol Plant **15** 473-497) with 2 µM silver thiosulphate, and nutrients and vitamins
30 as described by Linsmaier and Skoog (1965 Physiol Plant **18** 100-127). Cultures are maintained at 25°C with a 16h daily photoperiod. After approximately 40 days, subculturing is performed during which leaves are removed, and the shoots cut into

mononodal segments of approximately 8 mm length.

Shoot cultures of approximately 40 days maturity (5-6 cm height) are cut into 8 mm internodal segments which are placed into liquid LS-medium containing
 5 *Agrobacterium tumefaciens* transformed with pVICTOR IV GNG E35S *nagB* IV2' or pVICTOR IV GNG *rbc nagB* IV2' or pVICTOR IV GNG E35S *nagB*' ($A_{660} = 0.5$, pathlength 1 cm). Following incubation at room temperature for 30 minutes, the segments are dried by blotting on to sterile filter paper and transferred to LS agar (0.8% w/v containing 2 mg l⁻¹ 2,4-D and 500 µg l⁻¹ trans-zeatin. The explants are
 10 covered with filter paper, moistened with LS medium, and covered with a cloth for three days at 25°C. Following this treatment, the segments are washed with liquid LS medium containing 800 mg l⁻¹ carbenicillin, and transferred on to LS agar (0.8% w/v) containing 1 mg l⁻¹ trans-zeatin, 100 µg l⁻¹ gibberellic acid (GA3), with sucrose (eg 7.5 g l⁻¹) and glucosamine (eg 2.5 g l⁻¹) as the selection agent.

15

The segments are sub-cultured to fresh substrate each 3-4 weeks. In 3 to 4 weeks, shoots develop from the segments and the formation of new shoots continues for 3-4 months.

20 Rooting of regenerated shoots

The regenerated shoots are transferred to rooting substrate composed of LS-substrate, agar (8 g/l) and carbenicillin (800 mg/l).

25 The transgenic plants may be verified by performing a GUS assay on the co-introduced β -glucuronidase gene according to Hodal, L. *et al.* (Pl. Sci. (1992), 87: 115-122).

Alternatively, the transgenic genotype of the regenerated shoot may be verified by
 30 performing NPTII assays (Radke, S. E. *et al.*, Theor. Appl. Genet. (1988), 75: 685-694) or by performing PCR analysis according to Wang *et al* (1993, NAR 21 pp 4153-4154).

Transfer to soil

The newly rooted plants (height approx. 2-3 cms) are transplanted from rooting substrate to soil and placed in a growth chamber (21°C, 16 hour light 200-400uE/m²/sec). When the plants are well established they are transferred to the greenhouse, where they are grown until tubers had developed and the upper part of the plants are senescing.

Harvesting

10

The potatoes are harvested after about 3 months.

TRANSGENIC MAIZE PLANTS

15 Introduction

Since the first publication of production of transgenic plants in 1983 (Leemans, 1993 Biotechnology 11 s22), there have been numerous publications of production of transgenic plants including especially dicotyledon crop plants.

20

Until very recently there are very few reports on successful production of transgenic monocotyledonary crop plants. This relatively slow development within monocots are due to two causes. Firstly, until the early 1980s, efficient regeneration of plants from cultured cells and tissues of monocots had proven very difficult. This problem is ultimately solved by the culture of explants from immature and embryogenic tissue, which retain their morphogenic potential on nutrient media containing plant growth regulators. Secondly, the monocots are not a natural host for *Agrobacterium tumefaciens*, meaning that the successful developed techniques within the dicots using their natural vector *Agrobacterium tumefaciens* is unsuccessful for many years in the monocots.

30

Nevertheless, it is now possible to successfully transformation and produce fertile

transgenic plants of maize using methods such as: (1) Silicon Carbide Whiskers; (2) Particle Bombardment; (3) DNA Uptake by PEG treated protoplast; or (4) DNA Uptake in Electroporation of Tissue. Each of these methods - which are reviewed by Thompson (1995 *Euphytica* **85** pp 75-80) - may be used to prepare *inter alia* transgenic maize according to the present invention.

In particular, the particle Gun method has been successfully used for the transformation of monocots. However, EP-A-0604662 reports on a different method of transforming monocotyledons. The method comprises transforming cultured tissues of a monocotyledon under or after dedifferentiation with *Agrobacterium* containing a super binary vector as a selectable marker a hygromycin-resistant gene is used. Production of transgenic calli and plant is demonstrated using the hygromycin selection. This method may be used to prepare *inter alia* transgenic maize according to the present invention.

Subsequent to the method of EP-A-0604662, EP-A-0672752 reports on non-dedifferentiated immature embryos. In this regard, both hygromycin-resistance and PPT-resistance genes are used as the selectable marker, with PPT giving rise to 10% or more independent transformed plants. This method may be used to prepare *inter alia* transgenic maize according to the present invention.

To date, it would appear that transgenic maize plants can be successfully produced from easily-culturable varieties - such as the inbred line A188. In this regard, see the teachings of Ishida *et al* (1996 *Nature Biotechnology* **14** pp 745-750). The method disclosed by these workers may be used to prepare *inter alia* transgenic maize according to the present invention.

Vasil (1996 *Nature Biotechnology* **14** pp 702-703) presents a further review article on transformation of maize. Even though it is possible to prepare transformed maize by use of, for example, particle Gun mediated transformation, for the present studies the following protocol is adopted.

Plasmid construction

The disarmed *Agrobacterium tumefaciens* strain LBA 4404, containing the helper *vir* plasmid pRAL4404 (Hoekema *et al*, 1983 Nature **303** pp 179-180), is cultured on
 5 YMB agar ($K_2HPO_4 \cdot 3H_2O$ 660 mg l⁻¹, $MgSO_4$ 200 mg l⁻¹, NaCl 100 mg l⁻¹, mannitol 10 g l⁻¹, yeast extract 400 mg l⁻¹, 0.8% w/v agar, pH 7.0) containing 100 mg l⁻¹ rifampicin and 500 mg l⁻¹ streptomycin sulphate. Transformation with pVICTOR IV GNG E35S *nagB* IV2' or pVICTOR IV GNG *rbc nagB* IV2' or pVICTOR IV GNG E35S *nagB*' is accomplished using the freeze-thaw method of Holters *et al* (1978 Mol
 10 Gen Genet **163** 181-187) and transformants are selected on YMB agar containing 100 mg l⁻¹ rifampicin and 500 mg l⁻¹ streptomycin, and 50 mg l⁻¹ gentamycin sulphate.

Isolation and cocultivation of explants

15 Immature embryos of, for example, maize line A188 of the size between 1.5 to 2.5 mm are isolated and cocultivated with *Agrobacterium tumefaciens* strain LBA 4404 in N6-AS for 2-3 days at 25°C under illumination. Thereafter, the embryos are washed with sterilized water containing 250 mg/l of cefotaxime and transferred to an LS medium and 250 mg/l cefotaxime and glucosamine in concentrations of up to 100
 20 mg/l (the medium is hereafter called LSS1).

Conditions for the selection of transgenic plants

The explants are cultured for three weeks on LSS1 medium and then transferred to
 25 an LS medium containing glucosamine and cefotaxime. After three weeks on this medium, green shoots are isolated.

Rooting of transformed shoots

30 Transformed shoots are transferred to an MS medium containing 2 mg/l for rooting. After four weeks on this medium, plantlets are transferred to pots with sterile soil for acclimatisation.

TRANSGENIC GUAR PLANTS

Transformation of guar cotyledonary explants is performed according to Joersbo and Okkels (PCT/DK95/00221) using *Agrobacterium tumefaciens* LBA4404 harbouring
5 a suitable plasmid.

Other plants may be transformed in accordance with the present invention, such as other fruits, other vegetables, and other plants such as coffee plants, tea plants etc.

10 Other modifications of the present invention will be apparent to those skilled in the art.

SEQUENCES

(1) GENERAL INFORMATION:

- (i) APPLICANT:
 (A) NAME: DANISCO A/S
 (B) STREET: LANGEBROGADE 1
 (C) CITY: COPENHAGEN
 (D) STATE: COPENHAGEN K
 (E) COUNTRY: DENMARK
 (F) POSTAL CODE (ZIP): DK-1001

(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1088 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1091 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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36

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 Ile Asn Val Glu Ala Leu Arg Lys Gly Val Thr Thr Thr Ile Lys Phe
 995 1000 1005
 Ala Tyr Asp Thr Tyr Gln Tyr Val Phe Asp Gly Pro Phe Tyr Val Arg
 1010 1015 1020
 Ile Arg Asn Leu Thr Thr Ala Ser Lys Ile Asn Val Ser Ser Gly Ala
 1025 1030 1035 1040
 Gly Glu Glu Asp Met Thr Pro Thr Ser Ala Asn Ser Arg Ala Ala Leu
 1045 1050 1055
 Phe Ser Asp Gly Gly Val Gly Glu Tyr Trp Ala Asp Asn Asp Thr Ser
 1060 1065 1070
 Ser Leu Trp Met Lys Leu Pro Asn Leu Val Leu Gln Asp Ala Val Ile
 1075 1080 1085
 Thr Ile Thr
 1090

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1066 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met Ala Gly Phe Ser Asp Pro Leu Asn Phe Cys Lys Ala Glu Asp Tyr
 1 5 10 15
 Tyr Ser Val Ala Leu Asp Trp Lys Gly Pro Gln Lys Ile Ile Gly Val
 20 25 30
 Asp Thr Thr Pro Pro Lys Ser Thr Lys Phe Pro Lys Asn Trp His Gly
 35 40 45
 Val Asn Leu Arg Phe Asp Asp Gly Thr Leu Gly Val Val Gln Phe Ile
 50 55 60
 Arg Pro Cys Val Trp Arg Val Arg Tyr Asp Pro Gly Phe Lys Thr Ser
 65 70 75 80

Asp Glu Tyr Gly Asp Glu Asn Thr Arg Thr Ile Val Gln Asp Tyr Met
 85 90 95
 Ser Thr Leu Ser Asn Lys Leu Asp Thr Tyr Arg Gly Leu Thr Trp Glu
 100 105 110
 Thr Lys Cys Glu Asp Ser Gly Asp Phe Phe Thr Phe Ser Ser Lys Val
 115 120 125
 Thr Ala Val Glu Lys Ser Glu Arg Thr Arg Asn Lys Val Gly Asp Gly
 130 135 140
 Leu Arg Ile His Leu Trp Lys Ser Pro Phe Arg Ile Gln Val Val Arg
 145 150 155 160
 Thr Leu Thr Pro Leu Lys Asp Pro Tyr Pro Ile Pro Asn Val Ala Ala
 165 170 175
 Ala Glu Ala Arg Val Ser Asp Lys Val Val Trp Gln Thr Ser Pro Lys
 180 185 190
 Thr Phe Arg Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr
 195 200 205
 Val Leu Asp Ile Val Lys Pro Gly His Gly Glu Tyr Val Gly Trp Gly
 210 215 220
 Glu Met Gly Gly Ile Gln Phe Met Lys Glu Pro Thr Phe Met Asn Tyr
 225 230 235 240
 Phe Asn Phe Asp Asn Met Gln Tyr Gln Gln Val Tyr Ala Gln Gly Ala
 245 250 255
 Leu Asp Ser Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr Leu Asp
 260 265 270
 Val Asn Ser Asn Pro Glu His Lys Asn Ile Thr Ala Thr Phe Ile Asp
 275 280 285
 Asn Tyr Ser Gln Ile Ala Ile Asp Phe Gly Lys Thr Asn Ser Gly Tyr
 290 295 300
 Ile Lys Leu Gly Thr Arg Tyr Gly Gly Ile Asp Cys Tyr Gly Ile Ser
 305 310 315 320
 Ala Asp Thr Val Pro Glu Ile Val Arg Leu Tyr Thr Gly Leu Val Gly
 325 330 335
 Arg Ser Lys Leu Lys Pro Arg Tyr Ile Leu Gly Ala His Gln Ala Cys
 340 345 350
 Tyr Gly Tyr Gln Gln Glu Ser Asp Leu Tyr Ser Val Val Gln Gln Tyr
 355 360 365
 Arg Asp Cys Lys Phe Pro Leu Asp Gly Ile His Val Asp Val Asp Val
 370 375 380
 Gln Asp Gly Phe Arg Thr Phe Thr Thr Asn Pro His Thr Phe Pro Asn
 385 390 395 400
 Pro Lys Glu Met Phe Thr Asn Leu Arg Asn Gly Ile Lys Cys Ser
 405 410 415
 Thr Asn Ile Thr Pro Val Ile Ser Ile Asn Asn Arg Glu Gly Gly Tyr
 420 425 430
 Ser Thr Leu Leu Glu Gly Val Asp Lys Lys Tyr Phe Ile Met Asp Asp
 435 440 445
 Arg Tyr Thr Glu Gly Thr Ser Gly Asn Ala Lys Asp Val Arg Tyr Met
 450 455 460
 Tyr Tyr Gly Gly Gly Asn Lys Val Glu Val Asp Pro Asn Asp Val Asn
 465 470 475 480
 Gly Arg Pro Asp Phe Lys Asp Asn Tyr Asp Phe Pro Ala Asn Phe Asn
 485 490 495
 Ser Lys Gln Tyr Pro Tyr His Gly Gly Val Ser Tyr Gly Tyr Gly Asn
 500 505 510
 Gly Ser Ala Gly Phe Tyr Pro Asp Leu Asn Arg Lys Glu Val Arg Ile
 515 520 525
 Trp Trp Gly Met Gln Tyr Lys Tyr Leu Phe Asp Met Gly Leu Glu Phe
 530 535 540
 Val Trp Gln Asp Met Thr Thr Pro Ala Ile His Thr Ser Tyr Gly Asp
 545 550 555 560
 Met Lys Gly Leu Pro Thr Arg Leu Leu Val Thr Ser Asp Ser Val Thr
 565 570 575
 Asn Ala Ser Glu Lys Lys Leu Ala Ile Glu Thr Trp Ala Leu Tyr Ser
 580 585 590
 Tyr Asn Leu His Lys Ala Thr Trp His Gly Leu Ser Arg Leu Glu Ser
 595 600 605

Arg Lys Asn Lys Arg Asn Phe Ile Leu Gly Arg Gly Ser Tyr Ala Gly
 610 615 620
 Ala Tyr Arg Phe Ala Gly Leu Trp Thr Gly Asp Asn Ala Ser Asn Trp
 625 630 635 640
 Glu Phe Trp Lys Ile Ser Val Ser Gln Val Leu Ser Leu Gly Leu Asn
 645 650 655
 Gly Val Cys Ile Ala Gly Ser Asp Thr Gly Gly Phe Glu Pro Tyr Arg
 660 665 670
 Asp Ala Asn Gly Val Glu Glu Lys Tyr Cys Ser Pro Glu Leu Leu Ile
 675 680 685
 Arg Trp Tyr Thr Gly Ser Phe Leu Leu Pro Trp Leu Arg Asn His Tyr
 690 695 700
 Val Lys Lys Asp Arg Lys Trp Phe Gln Glu Pro Tyr Ser Tyr Pro Lys
 705 710 715 720
 His Leu Glu Thr His Pro Glu Leu Ala Asp Gln Ala Trp Leu Tyr Lys
 725 730 735
 Ser Val Leu Glu Ile Cys Arg Tyr Tyr Val Glu Leu Arg Tyr Ser Leu
 740 745 750
 Ile Gln Leu Leu Tyr Asp Cys Met Phe Gln Asn Val Val Asp Gly Met
 755 760 765
 Pro Ile Thr Arg Ser Met Leu Leu Thr Asp Thr Glu Asp Thr Thr Phe
 770 775 780
 Phe Asn Glu Ser Gln Lys Phe Leu Asp Asn Gln Tyr Met Ala Gly Asp
 785 790 795 800
 Asp Ile Leu Val Ala Pro Ile Leu His Ser Arg Lys Glu Ile Pro Gly
 805 810 815
 Glu Asn Arg Asp Val Tyr Leu Pro Leu Tyr His Thr Trp Tyr Pro Ser
 820 825 830
 Asn Leu Arg Pro Trp Asp Asp Gln Gly Val Ala Leu Gly Asn Pro Val
 835 840 845
 Glu Gly Gly Ser Val Ile Asn Tyr Thr Ala Arg Ile Val Ala Pro Glu
 850 855 860
 Asp Tyr Asn Leu Phe His Ser Val Val Pro Val Tyr Val Arg Glu Gly
 865 870 875 880
 Ala Ile Ile Pro Gln Ile Glu Val Arg Gln Trp Thr Gly Gln Gly
 885 890 895
 Ala Asn Arg Ile Lys Phe Asn Ile Tyr Pro Gly Lys Asp Lys Glu Tyr
 900 905 910
 Cys Thr Tyr Leu Asp Asp Gly Val Ser Arg Asp Ser Ala Pro Glu Asp
 915 920 925
 Leu Pro Gln Tyr Lys Glu Thr His Glu Gln Ser Lys Val Glu Gly Ala
 930 935 940
 Glu Ile Ala Lys Gln Ile Gly Lys Lys Thr Gly Tyr Asn Ile Ser Gly
 945 950 955 960
 Thr Asp Pro Glu Ala Lys Gly Tyr His Arg Lys Val Ala Val Thr Gln
 965 970 975
 Thr Ser Lys Asp Lys Thr Arg Thr Val Thr Ile Glu Pro Lys His Asn
 980 985 990
 Gly Tyr Asp Pro Ser Lys Glu Val Gly Asp Tyr Tyr Thr Ile Ile Leu
 995 1000 1005
 Trp Tyr Ala Pro Gly Phe Asp Gly Ser Ile Val Asp Val Ser Lys Thr
 1010 1015 1020
 Thr Val Asn Val Glu Gly Gly Val Glu His Gln Val Tyr Lys Asn Ser
 1025 1030 1035 1040
 Asp Leu His Thr Val Val Ile Asp Val Lys Glu Val Ile Gly Thr Thr
 1045 1050 1055
 Lys Ser Val Lys Ile Thr Cys Thr Ala Ala
 1060 1065

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1070 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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Met Ala Gly Leu Ser Asp Pro Leu Asn Phe Cys Lys Ala Glu Asp Tyr
1      5      10      15
Tyr Ala Ala Ala Lys Gly Trp Ser Gly Pro Gln Lys Ile Ile Arg Tyr
20      25      30
Asp Gln Thr Pro Pro Gln Gly Thr Lys Asp Pro Lys Ser Trp His Ala
35      40      45
Val Asn Leu Pro Phe Asp Asp Gly Thr Met Cys Val Val Gln Phe Val
50      55      60
Arg Pro Cys Val Trp Arg Val Arg Tyr Asp Pro Ser Val Lys Thr Ser
65      70      75      80
Asp Glu Tyr Gly Asp Glu Asn Thr Arg Thr Ile Val Gln Asp Tyr Met
85      90      95
Thr Thr Leu Val Gly Asn Leu Asp Ile Phe Arg Gly Leu Thr Trp Val
100      105      110
Ser Thr Leu Glu Asp Ser Gly Glu Tyr Tyr Thr Phe Lys Ser Glu Val
115      120      125
Thr Ala Val Asp Glu Thr Glu Arg Thr Arg Asn Lys Val Gly Asp Gly
130      135      140
Leu Lys Ile Tyr Leu Trp Lys Asn Pro Phe Arg Ile Gln Val Val Arg
145      150      155      160
Leu Leu Thr Pro Leu Val Asp Pro Phe Pro Ile Pro Asn Val Ala Asn
165      170      175
Ala Thr Ala Arg Val Ala Asp Lys Val Val Trp Gln Thr Ser Pro Lys
180      185      190
Thr Phe Arg Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr
195      200      205
Val Leu Asp Ile Ile Lys Pro Gly His Gly Glu Tyr Val Gly Trp Gly
210      215      220
Glu Met Gly Gly Ile Glu Phe Met Lys Glu Pro Thr Phe Met Asn Tyr
225      230      235      240
Phe Asn Phe Asp Asn Met Gln Tyr Gln Gln Val Tyr Ala Gln Gly Ala
245      250      255
Leu Asp Ser Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr Leu Asp
260      265      270
Val Asn Ser Asn Pro Glu His Lys Asn Ile Thr Ala Thr Phe Ile Asp
275      280      285
Asn Tyr Ser Gln Ile Ala Ile Asp Phe Gly Lys Thr Asn Ser Gly Tyr
290      295      300
Ile Lys Leu Gly Thr Arg Tyr Gly Gly Ile Asp Cys Tyr Gly Ile Ser
305      310      315      320
Ala Asp Thr Val Pro Glu Ile Val Arg Leu Tyr Thr Gly Leu Val Gly
325      330      335
Arg Ser Lys Leu Lys Pro Arg Tyr Ile Leu Gly Ala His Gln Ala Cys
340      345      350
Tyr Gly Tyr Gln Gln Glu Ser Asp Leu His Ala Val Val Gln Gln Tyr
355      360      365
Arg Asp Thr Lys Phe Pro Leu Asp Gly Leu His Val Asp Val Asp Phe
370      375      380
Gln Asp Asn Phe Arg Thr Phe Thr Thr Asn Pro Ile Thr Phe Pro Asn
385      390      395      400
Pro Lys Glu Met Phe Thr Asn Leu Arg Asn Asn Gly Ile Lys Cys Ser
405      410      415
Thr Asn Ile Thr Pro Val Ile Ser Ile Arg Asp Arg Pro Asn Gly Tyr
420      425      430
Ser Thr Leu Asn Glu Gly Tyr Asp Lys Lys Tyr Phe Ile Met Asp Asp
435      440      445

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Arg Tyr Thr Glu Gly Thr Ser Gly Asp Pro Gln Asn Val Arg Tyr Ser
 450 455 460
 Phe Tyr Gly Gly Gly Asn Pro Val Glu Val Asn Pro Asn Asp Val Trp
 465 470 475
 Ala Arg Pro Asp Phe Gly Asp Asn Tyr Asp Phe Pro Thr Asn Phe Asn
 485 490 495
 Cys Lys Asp Tyr Pro Tyr His Gly Gly Val Ser Tyr Gly Tyr Gly Asn
 500 505 510
 Gly Thr Pro Gly Tyr Tyr Pro Asp Leu Asn Arg Glu Glu Val Arg Ile
 515 520 525
 Trp Trp Gly Leu Gln Tyr Glu Tyr Leu Phe Asn Met Gly Leu Glu Phe
 530 535 540
 Val Trp Gln Asp Met Thr Thr Pro Ala Ile His Ser Ser Tyr Gly Asp
 545 550 555 560
 Met Lys Gly Leu Pro Thr Arg Leu Leu Val Thr Ala Asp Ser Val Thr
 565 570 575
 Asn Ala Ser Glu Lys Lys Leu Ala Ile Glu Ser Trp Ala Leu Tyr Ser
 580 585 590
 Tyr Asn Leu His Lys Ala Thr Phe His Gly Leu Gly Arg Leu Glu Ser
 595 600 605
 Arg Lys Asn Lys Arg Asn Phe Ile Leu Gly Arg Gly Ser Tyr Ala Gly
 610 615 620
 Ala Tyr Arg Phe Ala Gly Leu Trp Thr Gly Asp Asn Ala Ser Thr Trp
 625 630 635 640
 Glu Phe Trp Lys Ile Ser Val Ser Gln Val Leu Ser Leu Gly Leu Asn
 645 650 655
 Gly Val Cys Ile Ala Gly Ser Asp Thr Gly Gly Phe Glu Pro Ala Arg
 660 665 670
 Thr Glu Ile Gly Glu Glu Lys Tyr Cys Ser Pro Glu Leu Leu Ile Arg
 675 680 685
 Trp Tyr Thr Gly Ser Phe Leu Leu Pro Trp Leu Arg Asn His Tyr Val
 690 695 700
 Lys Lys Asp Arg Lys Trp Phe Gln Glu Pro Tyr Ala Tyr Pro Lys His
 705 710 715 720
 Leu Glu Thr His Pro Glu Leu Ala Asp Gln Ala Trp Leu Tyr Lys Ser
 725 730 735
 Val Leu Glu Ile Cys Arg Tyr Trp Val Glu Leu Arg Tyr Ser Leu Ile
 740 745 750
 Gln Leu Leu Tyr Asp Cys Met Phe Gln Asn Val Val Asp Gly Met Pro
 755 760 765
 Leu Ala Arg Ser Met Leu Leu Thr Asp Thr Glu Asp Thr Thr Phe Phe
 770 775 780
 Asn Glu Ser Gln Lys Phe Leu Asp Asn Gln Tyr Met Ala Gly Asp Asp
 785 790 795 800
 Ile Leu Val Ala Pro Ile Leu His Ser Arg Asn Glu Val Pro Gly Glu
 805 810 815
 Asn Arg Asp Val Tyr Leu Pro Leu Phe His Thr Trp Tyr Pro Ser Asn
 820 825 830
 Leu Arg Pro Trp Asp Asp Gln Gly Val Ala Leu Gly Asn Pro Val Glu
 835 840 845
 Gly Gly Ser Val Ile Asn Tyr Thr Ala Arg Ile Val Ala Pro Glu Asp
 850 855 860
 Tyr Asn Leu Phe His Asn Val Val Pro Val Tyr Ile Arg Glu Gly Ala
 865 870 875 880
 Ile Ile Pro Gln Ile Gln Val Arg Gln Trp Ile Gly Glu Gly Gly Pro
 885 890 895
 Asn Pro Ile Lys Phe Asn Ile Tyr Pro Gly Lys Asp Lys Glu Tyr Val
 900 905 910
 Thr Tyr Leu Asp Asp Gly Val Ser Arg Asp Ser Ala Pro Asp Asp Leu
 915 920 925
 Pro Gln Tyr Arg Glu Ala Tyr Glu Gln Ala Lys Val Glu Gly Lys Asp
 930 935 940
 Val Gln Lys Gln Leu Ala Val Ile Gln Gly Asn Lys Thr Asn Asp Phe
 945 950 955 960
 Ser Ala Ser Gly Ile Asp Lys Glu Ala Lys Gly Tyr His Arg Lys Val
 965 970 975

41

Ser Ile Lys Gln Glu Ser Lys Asp Lys Thr Arg Thr Val Thr Ile Glu
 980 985 990
 Pro Lys His Asn Gly Tyr Asp Pro Ser Lys Glu Val Gly Asn Tyr Tyr
 995 1000 1005
 Thr Ile Ile Leu Trp Tyr Ala Pro Gly Phe Asp Gly Ser Ile Val Asp
 1010 1015 1020
 Val Ser Gln Ala Thr Val Asn Ile Glu Gly Gly Val Glu Cys Glu Ile
 1025 1030 1035 1040
 Phe Lys Asn Thr Gly Leu His Thr Val Val Val Asn Val Lys Glu Val
 1045 1050 1055
 Ile Gly Thr Thr Lys Ser Val Lys Ile Thr Cys Thr Thr Ala
 1060 1065 1070

SEQ. ID. NO. 5

SEQUENCE TYPE: ENZYME

MOLECULE TYPE: AMINO ACID

ORIGINAL SOURCE: ALGAL

SEQUENCE LENGTH: 1092 AMINO ACIDS

SEQUENCE:

	5	10	15
1	Met	Phe	Pro
16	Ser	Thr	Phe
31	Ser	Ala	Leu
46	Tyr	Asn	Val
61	Ser	Ile	Gly
76	Asp	Tyr	Ile
91	Trp	Tyr	Ala
106	Thr	Ala	Thr
121	Tyr	Ser	Asn
136	Asn	Asn	Ser
151	Asp	Val	Ser
166	Arg	Asn	Gln
181	Ser	Pro	Glu
196	Ile	Ile	Tyr
211	Tyr	Leu	Val
226	Asn	Lys	Cys
241	Ala	Ile	Ala
256	Asn	Glu	Lys
271	Glu	Gln	Gly
286	Ala	Met	Thr
301	Asp	Val	Val
316	Pro	Met	Tyr
331	Gly	Thr	Ser
346	Ser	Gln	Ser
361	Gln	Glu	Asn
376	Gln	His	Phe
391	Lys	Ala	Phe
406	Leu	Asn	Lys
421	Phe	Gln	Gly
436	Pro	Ala	Gly
451	Tyr	Gln	Asp
466	Asp	Met	Gln
481	Trp	Ser	Ala
496	Ser	Val	Phe
511	Asn	Val	Thr
526	Glu	Val	Asn
541	Asp	Ser	Leu
556	Gly	Asp	Ala
571	Cys	Asp	Ala
586	Trp	Trp	Gly
601	Phe	Val	Trp
616	Leu	Gly	Asp
631	Trp	Pro	Asn
646	Ser	Tyr	His
661	Glu	Tyr	Gly

42

676 Tyr Thr Leu Cys Glu Ser Thr Arg Arg Glu Gly Ile Val Gly Asn
 691 Ala Asp Ser Leu Thr Lys Phe Arg Arg Ser Tyr Ile Ile Ser Arg
 706 Gly Gly Tyr Ile Gly Asn Gln His Phe Gly Gly Met Trp Val Gly
 721 Asp Asn Ser Ala Thr Glu Ser Tyr Leu Gln Met Met Leu Ala Asn
 736 Ile Ile Asn Met Asn Met Ser Cys Leu Pro Leu Val Gly Ser Asp
 751 Ile Gly Gly Phe Thr Gln Tyr Asn Asp Ala Gly Asp Pro Thr Pro
 766 Glu Asp Leu Met Val Arg Phe Val Gln Ala Gly Cys Leu Leu Pro
 781 Trp Phe Arg Asn His Tyr Asp Arg Trp Ile Glu Ser Lys Lys His
 796 Gly Lys Lys Tyr Gln Glu Leu Tyr Met Tyr Pro Gly Gln Lys Asp
 811 Thr Leu Lys Lys Phe Val Glu Phe Arg Tyr Arg Trp Gln Glu Val
 826 Leu Tyr Thr Ala Met Tyr Gln Asn Ala Thr Thr Gly Glu Pro Ile
 841 Ile Lys Ala Ala Pro Met Tyr Asn Asn Asp Val Asn Val Tyr Lys
 856 Ser Gln Asn Asp His Phe Leu Leu Gly Gly His Asp Gly Tyr Arg
 871 Ile Leu Cys Ala Pro Val Val Arg Glu Asn Ala Thr Ser Arg Glu
 886 Val Tyr Leu Pro Val Tyr Ser Lys Trp Phe Lys Phe Gly Pro Asp
 901 Phe Asp Thr Lys Pro Leu Glu Asn Glu Ile Gln Gly Gly Gln Thr
 916 Leu Tyr Asn Tyr Ala Ala Pro Leu Asn Asp Ser Pro Ile Phe Val
 931 Arg Glu Gly Thr Ile Leu Pro Thr Arg Tyr Thr Leu Asp Gly Val
 946 Asn Lys Ser Ile Asn Thr Tyr Thr Asp Asn Asp Pro Leu Val Phe
 961 Glu Leu Phe Pro Leu Glu Asn Asn Gln Ala His Gly Leu Phe Tyr
 976 His Asp Asp Gly Gly Val Thr Thr Asn Ala Glu Asp Phe Gly Lys
 991 Tyr Ser Val Ile Ser Val Lys Ala Ala Gln Glu Gly Ser Gln Met
 1006 Ser Val Lys Phe Asp Asn Glu Val Tyr Glu His Gln Trp Gly Ala
 1021 Ser Phe Tyr Val Arg Val Arg Asn Met Gly Ala Pro Ser Asn Ile
 1036 Asn Val Ser Ser Gln Ile Gly Gln Gln Asp Met Gln Gln Ser Ser
 1051 Val Ser Ser Arg Ala Gln Met Phe Thr Ser Ala Asn Asp Gly Glu
 1066 Tyr Trp Val Asp Gln Ser Thr Asn Ser Leu Trp Leu Lys Leu Pro
 1081 Gly Ala Val Ile Gln Asp Ala Ala Ile Thr Val Arg

Number of amino acid residues: 1092

Amino acid composition (including the signal sequence):

64 Ala	14 Cys	18 His	33 Met	56 Thr
48 Arg	55 Gln	45 Ile	49 Phe	22 Trp
89 Asn	49 Glu	65 Leu	59 Pro	67 Tyr
73 Asp	94 Gly	46 Lys	73 Ser	73 Val

SEQ. ID. NO. 6

SEQUENCE TYPE: ENZYME

MOLECULE TYPE: AMINO ACID

ORIGINAL SOURCE: ALGAL

SEQUENCE LENGTH: 570 AMINO ACIDS

SEQUENCE:

	5	10	15
1 Met Thr Asn Tyr Asn Tyr Asp Asn Leu Asn Tyr Asn Gln Pro Asp			
16 Leu Ile Pro Pro Gly His Asp Ser Asp Pro Asp Tyr Tyr Ile Pro			
31 Met Tyr Phe Ala Ala Pro Trp Val Ile Ala His Gly Tyr Arg Gly			
46 Thr Ser Asp Gln Tyr Ser Tyr Gly Trp Phe Leu Asp Asn Val Ser			
61 Gln Ser Tyr Thr Asn Thr Gly Asp Asp Ala Trp Ala Gly Gln Lys			
76 Asp Leu Ala Tyr Met Gly Ala Gln Cys Gly Pro Phe Asp Gln His			
91 Phe Val Tyr Glu Ala Gly Asp Gly Leu Glu Asp Val Val Thr Ala			
106 Phe Ser Tyr Leu Gln Gly Lys Glu Tyr Glu Asn Gln Gly Leu Asn			
121 Ile Arg Ser Ala Met Pro Pro Lys Tyr Val Phe Gly Phe Phe Gln			
136 Gly Val Phe Gly Ala Thr Ser Leu Leu Arg Asp Asn Leu Pro Ala			
151 Gly Glu Asn Asn Val Ser Leu Glu Glu Ile Val Glu Gly Tyr Gln			
166 Asn Gln Asn Val Pro Phe Glu Gly Leu Ala Val Asp Val Asp Met			
181 Gln Asp Asp Leu Arg Val Phe Thr Thr Arg Pro Ala Phe Trp Thr			
196 Ala Asn Lys Val Gly Glu Gly Asp Pro Asn Asn Lys Ser Val			
211 Phe Glu Trp Ala His Asp Arg Gly Leu Val Cys Gln Thr Asn Val			
226 Thr Cys Phe Leu Lys Asn Glu Lys Asn Pro Tyr Glu Val Asn Gln			
241 Ser Leu Arg Glu Lys Gln Leu Tyr Thr Lys Ser Asp Ser Leu Asp			
256 Asn Ile Asp Phe Gly Thr Thr Pro Asp Gly Pro Ser Asp Ala Tyr			
271 Ile Gly His Leu Asp Tyr Gly Gly Gly Val Glu Cys Asp Ala Leu			
286 Phe Pro Asp Trp Gly Arg Pro Asp Val Ala Gln Trp Trp Gly Asp			

301 Asn Tyr Lys Lys Leu Phe Ser Ile Gly Leu Asp Phe Val Trp Gln
 316 Asp Met Thr Val Pro Ala Met Met Pro His Arg Leu Gly Asp Pro
 331 Val Gly Thr Asn Ser Gly Glu Thr Ala Pro Gly Trp Pro Asn Asp
 346 Lys Asp Pro Ser Asn Gly Arg Tyr Asn Trp Lys Ser Tyr His Pro
 361 Gln Val Leu Val Thr Asp Met Arg Tyr Asp Asp Tyr Gly Arg Asp
 376 Pro Ile Val Thr Gln Arg Asn Leu His Ala Tyr Thr Leu Cys Glu
 391 Ser Thr Arg Arg Glu Gly Ile Val Gly Asn Ala Asp Ser Leu Thr
 406 Lys Phe Arg Arg Ser Tyr Ile Ile Ser Arg Gly Gly Tyr Ile Gly
 421 Asn Gln His Phe Gly Gly Met Trp Val Gly Asp Asn Ser Ser Thr
 436 Glu Asp Tyr Leu Ala Met Met Val Ile Asn Val Ile Asn Met Asn
 451 Met Ser Gly Val Pro Leu Val Gly Ser Asp Ile Gly Gly Phe Thr
 466 Glu His Asp Lys Arg Asn Pro Cys Thr Pro Asp Leu Met Met Arg
 481 Phe Val Gln Ala Gly Cys Leu Leu Pro Trp Phe Arg Asn His Tyr
 496 Asp Arg Trp Ile Glu Ser Lys Lys His Gly Lys Asn Tyr Gln Glu
 511 Leu Tyr Met Tyr Arg Asp His Leu Asp Ala Leu Arg Ser Phe Val
 526 Glu Leu Arg Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr
 541 Gln Asn Ala Leu Asn Gly Lys Pro Ile Ile Lys Thr Val Ser Met
 556 Tyr Asn Asn Asp Met Asn Val Lys Asp Ala Gln Asn Asp His Phe

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3267 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATGTTTTCAA	CCCTTGCGTT	TGTCGCACCT	AGTGCCTGG	GAGCCAGTAC	CTTCGTAGGG	60
GCGGAGGTCA	GGTCAAATGT	TCGTATCCAT	TCCGCTTTTC	CAGCTGTGCA	CACAGCTACT	120
CGCAAAACCA	ATCGCCTCAA	TGTATCCATG	ACCGCATTGT	CCGACAAACA	AACGGCTACT	180
GCGGGTAGTA	CAGACAATCC	GGACGGTATC	GACTACAAGA	CCTACGATTA	CGTCGGAGTA	240
TGGGGTTTCA	GCCCCCTCTC	CAACACGAAC	TGGTTTGCTG	CCGGCTCTTC	TACCCCGGGT	300
GGCATCACTG	ATTGGACGGC	TACAATGAAT	GTCAACTTCG	ACCGTATCGA	CAATCCGTCC	360
ATCACTGTCC	AGCATCCCGT	TCAGGTTCCAG	GTCACTGTCAT	ACAACAACAA	CAGCTACAGG	420
GTTTCGTTCA	ACCCTGATGG	CCCTATTTCGT	GATGTGACTC	GTGGGCCTAT	CCTCAAGCAG	480
CAACTAGATT	GGATTCTGAAC	GCAGGAGCTG	TCAGAGGGAT	GTGATCCCGG	AATGACTTTC	540
ACATCAGAAG	GTTTCTTGAC	TTTTGAGACC	AAGGATCTAA	GCGTCATCAT	CTACGGAAAT	600
TTCAAGACCA	GAGTTACGAG	AAAGTCTGAC	GGCAAGGTCA	TCATGGAAAA	TGATGAAGTT	660
GGAAGTGCAT	CGTCCGGGAA	CAAGTGCCGG	GGATTGATGT	TCGTTGATAG	ATTATACGGT	720
AACGCTATCG	CTTCCGTCAA	CAAGAATTC	CGCAACGACG	CGGTCAAGCA	GGAGGGATTTC	780
TATGGTGCAG	GTGAAGTCAA	CTGTAAGTAC	CAGGACACCT	ACATCTTAGA	ACGCACTGGA	840
ATCGCCATGA	CAAATTACAA	CTACGATAAC	TTGAACTATA	ACCAGTGGGA	CCTTAGACCT	900
CCGCATCATG	ATGGTGCCCT	CAACCCAGAC	TATTATATTC	CAATGTACTA	CGCAGCACCT	960
TGGTTGATCG	TTAATGGATG	CGCCGGTACT	TCGGAGCAGT	ACTCGTATGG	ATGGTTTCATG	1020
GACAATGTCT	CTCAATCTTA	CATGAATACT	GGAGATACTA	CCTGGAATTC	TGGACAAGAG	1080
GACCTGGCAT	ACATGGGCGC	GCAGTATGGA	CCATTTGACC	AACATTTTGT	TTACGGTGCT	1140
GGGGGTGGGA	TGGAATGTGT	GGTCACAGCG	TTCTCTCTTC	TACAAGGCAA	GGAGTTCGAG	1200
AACCAAGTTC	TCAACAAACG	TTCAAGTAATG	CCTCCGAAAT	ACGTCTTTGG	TTTCTTCCAG	1260
GGTGTTCG	GGACTTCTTC	CTTGTGAGA	GCGCATATGC	CAGCAGGTGA	GAACAACATC	1320
TCAGTCTGAAG	AAATTGTAGA	AGGTTATCAA	AACAACAATT	TCCCTTTTGA	GGGGCTCGCT	1380
GTGGACGTGG	ATATGCAAGA	CAACTTGCGG	GTGTTACCA	CGAAGGCGGA	ATTTTGGACC	1440
GCAACAGGG	TGGGTACTGG	CGGGGATCCA	AACAACCGAT	CGGTTTTTGA	ATGGGCACAT	1500
GACAAAGGCC	TTGTTTGTC	GACAAATATA	ACTTGCTTCC	TGAGGAATGA	TAACGAGGGG	1560
CAAGACTACG	AGGTCAATCA	GACGTTAAGG	GAGAGGCGAGT	TGTACACGAA	GAACGACTCC	1620
CTGACGGGTA	CGGATTTTGG	AATGACCGAC	GACGGCCCCA	GCGATGCGTA	CATCGGTCAT	1680
CTGGACTATG	GGGGTGGAGT	AGAATGTGAT	GCACTTTTCC	CAGACTGGGG	ACGGCCTGAC	1740
GTGGCCGAAT	GGTGGGGAAA	TAACATAAAG	AACTGTTTCA	GCAATGGTCT	CGACTTCGTC	1800
TGGCAAGACA	TGACTGTTCC	AGCAATGATG	CCGCACAAAA	TTGGCGATGA	CATCAATGTG	1860
AAACCGGATG	GGAAATGGCC	GAATGCGGAC	GATCCGCTCA	ATGGACAATA	CAACTGGAAG	1920
ACGTACCATC	CCCAAGTGCT	TGTAAGTATG	ATGCGTTATG	AGAATCATGG	TCGGGAACCG	1980
ATGGTCACTC	AACGCAACAT	TCATGCGTAT	ACACTGTGCG	AGTCTACTAG	GAAGGAAGGG	2040
ATCGTGGAAA	ACGCAGACAC	TCTAACGAAG	TTCCGCCGTA	GCTACATTAT	CAGTCGTGGT	2100
GTTTACATTG	GTAACAGCA	TTTCGGGGT	ATGTGGGTGG	GAGACAACCTC	TACTACATCA	2160

AACTACATCC	AAATGATGAT	TGCCAACAAAT	ATTAACATGA	ATATGTCTTG	CTTGCCTCTC	2220
GTCGGGCTCCG	ACATTGGAGG	ATTCACCTCA	TACGACAATG	AGAATCAGCG	AACGCCGTGT	2280
ACCGGGGACT	TGATGGTGAG	GTATGTGCAG	GCGGGCTGCC	TGTTGCCGTG	GTTTCAGGAAC	2340
CACTATGATA	GGTGGATCGA	GTCCAAGGAC	CACGGAAAGG	ACTACCAGGA	GCTGTACATG	2400
TATCCGAATG	AAATGGATAC	GTTGAGGAAG	TTCGTTGAAT	TCCGTTATCG	CTGGCAGGAA	2460
GTGTTGTACA	CGGCCATGTA	CCAGAAATGCG	GCTTTCGGAA	AGCCGATTAT	CAAGGCTGCT	2520
TCGATGTACA	ATAACGACTC	AAACGTTTCG	AGGGCGCAGA	ACGATCATTT	CCTTCTTGGT	2580
GGACATGATG	GATATCGCAT	TCTGTGCGCG	CCTGTTGTGT	GGGAGAATTC	GACCGAACGC	2640
GAATTGTACT	TGCCCGTGCT	GACCCAATGG	TACAAATTCG	GTCCCGACTT	TGACACCAAG	2700
CCTCTGGAAG	GAGCGATGAA	CGGAGGGGAC	CGAATTTACA	ACTACCCTGT	ACCGCAAAGT	2760
GAATCACCAA	TCTTCGTGAG	AGAAGGTGCG	ATTCTCCCTA	CCCGCTACAC	GTTGAACGGT	2820
GAAAAACAAAT	CATTGAACAC	GTACACGGAC	GAAGATCCGT	TGGTGTTTGA	AGTATTCCCC	2880
CTCGGAAACA	ACCGTGCCGA	CGGTATGTGT	TATCTTGATG	ATGGCGGTGT	GACCACCAAT	2940
GCTGAAGACA	ATGGCAAGTT	CTCTGTCGTC	AAGGTGGCAG	CGGAGCAGGA	TGGTGGTACG	3000
GAGACGATAA	CGTTTACGAA	TGATTGCTAT	GAGTACGTTT	TCGGTGGACC	GTTCTACGTT	3060
CGAGTGCGCG	GCGCTCAGTC	GCCGTCGAAC	ATCCACGTGT	CTTCTGGAGC	GGGTTCTCAG	3120
GACATGAAGG	TGAGCTCTGC	CACTTCCAGG	GCTGCGCTGT	TCAATGACGG	GGAGAACGGT	3180
GATTTCTGGG	TTGACCAGGA	GACAGATTCT	CTGTGGCTGA	AGTTGCCCAA	CGTTGTTCTC	3240
CCGGACGCTG	TGATCACAAT	TACCTAA				3267

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3276 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ATGTATCCAA	CCCTCACCTT	CGTGGCGCCT	AGTGCGCTAG	GGGCCAGAAC	TTTCACGTGT	60
GTGGGCATTT	TTAGGTGACA	CATTCTTATT	CATTCCGTTG	TTCCAGCGGT	GCGTCTAGCT	120
GTGCGCAAAA	GCAACCGCCT	CAATGTATCC	ATGTCCGCTT	TGTTCCGACAA	ACCGACTGCT	180
GTTACTGGAG	GGAAGGACAA	CCCGGACAAAT	ATCAATTACA	CCACTTATGA	CTACGTCCCT	240
GTGTGGCGCT	TCGACCCCTT	CAGCAATACG	AACTGGTTTG	CTGCCGGATC	TTCCACTCCC	300
GGCGATATTG	ACGACTGGAC	GGCGACAATG	AATGTGAAC	TCGACCGTAT	CGACAATCCA	360
TCCTTCACTC	TCGAGAAACC	GGTTCAGGTT	CAGGTCACGT	CATACAAGAA	CAATTGTTTC	420
AGGGTTCGCT	TCAACCTTGA	TGGTCCTATT	CGCGATGTGG	ATCGTGGGCC	TATCCTCCAG	480
CAGCAACTAA	ATTGGATCCG	GAAGCAGGAG	CAGTCGAAGG	GGTTTGATCC	TAAGATGGGC	540
TTACAAAAG	AAGGTTTCTT	GAAATTTGAG	ACCAAGGATC	TGAACGTTAT	CATATATGGC	600
AATTTTAAGA	CTAGAGTTAC	GAGGAAGAGG	GATGGAAGAA	GGATCATGGA	GAATAATGAA	660
GTGCCGGCAG	GATCGTTAGG	GAACAAGTGC	CGGGGATTGA	TGTTTGTCGA	CAGGTTGTAC	720
GGCACTGCCA	TCGCTTCCGT	TAATGAAAAT	TACCGCAACG	ATCCCGACAG	GAAAGAGGGG	780
TTCTATGGTG	CAGGAGAAAG	AAACTGCGAG	TTTTGGGACT	CCGAACAAAA	CAGGAACAAG	840
TACATCTTAG	AACGAACTGG	AATCGCCATG	ACAAATTACA	ATTATGACAA	CTATAACTAC	900
AACCACTCAG	ATCTTATTGC	TCCAGGATAT	CCTTCCGACC	CGAACTTCTA	CATTCCCATG	960
TATTTTGCAG	CACCTTGGGT	AGTTGTTAAG	GGATGCAGTG	GCAACAGCGA	TGAACAGTAC	1020
TCGTACGGAT	GGTTTATGGA	TAATGTCTCC	CAAACTTACA	TGAATACTGG	TGGTACTTCC	1080
TGGAACCTGTG	GAGAGGAGAA	CTTGGCATA	ATGGGAGCAC	AGTGCGGTCC	ATTTGACCAA	1140
CATTTTGTGT	ATGGTGATGG	AGATGGTCTT	GAGGATGTTG	TCCAAGCGTT	CTCTCTTCTG	1200
CAAGGCAAAAG	AGTTTGAGAA	CCAAGTTCTG	AACAAACGTG	CCGTAATGCC	TCCGAAATAT	1260
GTGTTTGGTT	ACTTTCAGGG	AGTCTTTGGG	ATTGCTTCTT	TGTTGAGAGA	GCAAAGACCA	1320
GAGGGTGGTA	ATAACATCTC	TGTTCAAGAG	ATTGTGGAAG	GTTACCAAAG	CAATAACTTC	1380
CCTTTAGAGG	GGTTAGCCGT	AGATGTGGAT	ATGCAACAAG	ATTTGCGCGT	GTTCAACACG	1440
AAGATTGAAT	TTTGGACGGC	AAATAAGGTA	GGCACCGGGG	GAGACTCGAA	TAACAAGTCG	1500
GTGTTTGAAT	GGGCACATGA	CAAAGGCCCT	GTATGTCAGA	CGAATGTTAC	TTGCTTCTTG	1560
AGAAACGACA	ACGGCGGGGC	AGATTACGAA	GTCAATCAGA	CATTGAGGGA	GAAGGGTTTG	1620
TACACGAAGA	ATGACTCACT	GACGAACACT	AACCTCGGAA	CTACCAACGA	CGGGCCGAGC	1680
GATGCGTACA	TTGGACATCT	GGACTATGGT	GGCGGAGGGA	ATTGTGATGC	ACTTTTCCCA	1740
GACTGGGGTC	GACCGGGTGT	GGCTGAATGG	TGGGGTGATA	ACTACAGCAA	GCTCTTCAAA	1800
ATTGGTCTGG	ATTTCTGCTG	GCAAGACATG	ACAGTTCCAG	CTATGATGCC	ACACAAGGTT	1860
GGCGACGCAG	TCGATACGAG	ATCACCTTAC	GGCTGGCCGA	ATGAGAATGA	TCCTTCGAAC	1920
GGACGATACA	ATTGGAAATC	TTACCATCCA	CAAGTTCTCG	TAAGTGATAT	GCGATATGAG	1980
AATCATGGAA	GGGAACCGAT	GTTCACTCAA	CGCAATATGC	ATGCGTACAC	ACTCTGTGAA	2040

TCTACGAGGA	AGGAAGGGAT	TGTTGCAAAT	GCAGACACTC	TAACGAAGTT	CCGCCGCAGT	2100
TATATTATCA	GTCGTGGAGG	TTACATTGGC	AACCAGCATT	TTGGAGGAAT	GTGGGTTGGA	2160
GACAACTCTT	CCTCCCAAAG	ATACCTCCAA	ATGATGATCG	CGAACATCGT	CAACATGAAC	2220
ATGTCTTGCC	TTCCACTAGT	TGGGTCCGAC	ATTGGAGGTT	TTACTTCGTA	TGATGGACGA	2280
AACGTGTGTC	CCGGGGATCT	AATGGTAAGA	TTCGTGCAGG	CGGGTTGCTT	ACTACCGTGG	2340
TTCAGAAACC	ACTATGGTAG	GTTGGTCGAG	GGCAAGCAAG	AGGGAAAATA	CTATCAAGAA	2400
CTGTACATGT	ACAAGGACGA	GATGGCTACA	TTGAGAAAAT	TCATTGAATT	CCGTTACCGC	2460
TGGCAGGAGG	TGTTGTACAC	TGCTATGTAC	CAGAATGCGG	CTTTCGGGAA	ACCGATTATC	2520
AAGGCAGCTT	CCATGTACGA	CAACGACAGA	AACGTTCCGG	GCGCACAGGA	TGACCACTTC	2580
CTTCTCGGCG	GACACGATGG	ATATCGTATT	TTGTGTGCAC	CTGTTGTGTG	GGAGAATACA	2640
ACCAGTCGCG	ATCTGTACTT	GCCTGTGCTG	ACCAAATGGT	ACAAATTCGG	CCCTGACTAT	2700
GACACCAAGC	GCCTGGATTG	TGCGTTGGAT	GGAGGGCAGA	TGATTAAGAA	CTATTCTGTG	2760
CCACAAAGCG	ACTCTCCGAT	ATTTGTGAGG	GAAGGAGCTA	TTCTCCCTAC	CCGCTACACG	2820
TTGGACGGTT	CGAACAAGTC	AATGAACACG	TACACAGACA	AAGACCCGTT	GGTGTGTTGAG	2880
GTATTCCTC	TTGGAAACAA	CCGTGCCGAC	GGTATGTGTT	ATCTTGATGA	TGGCGGTATT	2940
ACTACAGATG	CTGAGGACCA	TGGCAAATTC	TCTGTTATCA	ATGTCGAAGC	CTTACGGAAA	3000
GGTGTACGA	CGACGATCAA	GTTTGCGTAT	GACACTTATC	AATACGTATT	TGATGGTCCA	3060
TTCTACGTTT	GAATCCGTAA	TCTTACGACT	GCATCAAAAA	TTAACGTGTC	TTCTGGAGCG	3120
GGTGAAGAGG	ACATGACACC	GACCTCTGCG	AACTCGAGGG	CAGCTTTGTT	CAGTGATGGA	3180
GGTGTGAGG	AATACTGGGC	TGACAATGAT	ACGTCTTCTC	TGTGGATGAA	GTTGCCAAAC	3240
CTGGTTCTGC	AAGACGCTGT	GATTACCATT	ACGTAG			3276

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 3201 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

ATGGCAGGAT	TTTCTGATCC	TCTCAACTTT	TGCAAAGCAG	AAGACTACTA	CAGTGTGCG	60
CTAGACTGGA	AGGGCCCTCA	AAAAATCATT	GGAGTAGACA	CTACTCCTCC	AAAGAGCACC	120
AAGTTCCCCA	AAAACCTGGCA	TGGAGTGAAC	TTGAGATTTC	ATGATGGGAC	TTTAGGTGTG	180
GTTTCAGTTCA	TTAGGCCGTG	CGTTTGGAGG	GTTAGATACG	ACCCTGGTTT	CAAGACCTCT	240
GACGAGTATG	GTGATGAGAA	TACGAGGACA	ATTGTGCAAG	ATTATATGAG	TACTCTGAGT	300
AATAAAATTGG	ATACTTATAG	AGGTCTTACG	TGGGAAACCA	AGTGTGAGGA	TTCGGGAGAT	360
TTCTTTACCT	TCTCATCCAA	GGTCACCGCC	GTTGAAAAAT	CCGAGCGGAC	CCGCAACAAG	420
GTCCGGCGATG	GCCTCAGAAT	TCACCTATGG	AAAAGCCCTT	TCCGCATCCA	AGTAGTGCGC	480
ACCTTGACCC	CTTTGAAGGA	TCCTTACCCC	ATTCCAAATG	TAGCCGCAGC	CGAAGCCCGT	540
GTGTCCGACA	AGGTCGTTTG	GCAAACGTCT	CCCAAGACAT	TCAGAAAGAA	CCTGCATCCG	600
CAACACAAGA	TGCTAAAGGA	TACAGTTCTT	GACATTGTCA	AACCTGGACA	TGGCGAGTAT	660
GTGGGGTGGG	GAGAGATGGG	AGGTATCCAG	TTTATGAAGG	AGCCAACATT	CATGAACAT	720
TTTAACTTCG	ACAATATGCA	ATACCAGCAA	GTCTATGCCC	AAGGTGCTCT	CGATTCTCGC	780
GAGCCACTGT	ACCACTCGGA	TCCCTTCTAT	CTTGATGTGA	ACTCCAACCC	GGAGCACAAAG	840
AATATCACGG	CAACCTTTAT	CGATAACTAC	TCTCAAATTG	CCATCGACTT	TGGAAAGACC	900
AACTCAGGCT	ACATCAAGCT	GGGAACCAGG	TATGGTGGTA	TCGATTGTTA	CGGTATCAGT	960
GCGGATACGG	TCCCGGAAAT	TGTACGACTT	TATACAGGTC	TTGTTGGACG	TTCAAAGTTG	1020
AAGCCCAGAT	ATATTCTCGG	GGCCCATCAA	GCCTGTTATG	GATACCAACA	GGAAAGTGAC	1080
TTGTATTCTG	TGGTCCAGCA	GTACCGTGAC	TGTAATTTTC	CACCTGACGG	GATTACACGT	1140
GATGTCGATG	TTCAGGACGG	CTTCAGAACT	TTCACCACCA	ACCCACACAC	TTTCCCTAAC	1200
CCCAAGAGAG	TGTTTACTAA	CTTGAGGAAT	AATGGAATCA	AGTGCTCCAC	CAATATCACT	1260
CCTGTTATCA	GCATTAAACAA	CAGAGAGGGT	GGATACAGTA	CCCTCCTTGA	GGGAGTTGAC	1320
AAAAAATACT	TTATCATGGA	CGACAGATAT	ACCGAGGGAA	CAAGTGGGAA	TGCGAAGGAT	1380
GTTCCGTACA	TGTACTACGG	TGGTGGTAAT	AAGGTTGAGG	TCGATCCTAA	TGATGTTAAT	1440
GGTCGGCCAG	ACTTTAAAGA	CAACTATGAC	TTCCCCGCGA	ACTTCAACAG	CAACAATAC	1500
CCCTATCATG	GTGGTGTGAG	CTACGGTTAT	GGGAACGGTA	GTGCAGGTTT	TTACCCGGAC	1560
CTCAACAGAA	AGGAGGTTTC	TATCTGGTGG	GGAATGCAGT	ACAAGTATCT	CTTCGATATG	1620
GGAAGTGAAT	TTGTGTGGCA	AGACATGACT	ACCCACAGCA	TCCACACATC	ATATGGAGAC	1680
ATGAAAGGGT	TGCCCAACCG	TCTACTCGTC	ACCTCAGACT	CCGTCAACCA	TGCCTCTGAG	1740
AAAAAGCTCG	CAATTGAAAC	TTGGGCTCTC	TACTCCTACA	ATCTCCACAA	AGCAACTTGG	1800
CATGGTCTTA	GTCTGTCTGA	ATCTCGTAAG	AACAAACGAA	ACTTCATCCT	CGGGCGTGGA	1860
AGTTATGCCG	GAGCCTATCG	TTTTGTGGT	CTCTGGACTG	GGGATAATGC	AAGTAACTGG	1920
GAATTCTGGA	AGATATCGGT	CTCTCAAGTT	CTTCTCTG	GCCTCAATGG	TGTGTGCATC	1980

GCGGGGTCTG	ATACGGGTGG	TTTTGAACCC	TACCGTGATG	CAAATGGGGT	CGAGGAGAAA	2040
TACTGTAGCC	CAGAGCTACT	CATCAGGTGG	TATACTGGTT	CATTCTCTTT	GCCGTGGCTC	2100
AGGAACCAAT	ATGTCAAAAA	GGACAGGAAA	TGGTTCCAGG	AACCATACTC	GTACCCCAAG	2160
CATCTTGAAA	CCCATCCAGA	ACTCGCAGAC	CAAGCATGGC	TCTATAAATC	CGTTTTGGAG	2220
ATCTGTAGGT	ACTATGTGGA	GCTTAGATAC	TCCCTCATCC	AACTACTTTA	CGACTGCATG	2280
TTTCAAAACG	TAGTCGACGG	TATGCCAATC	ACCAGATCTA	TGCTCTTGAC	CGATACTGAG	2340
GATACCACCT	TCTTCAACGA	GAGCCAAAAG	TTCTCTGACA	ACCAATATAT	GGCTGGTGAC	2400
GACATTCTTG	TTGCACCCAT	CCTCCACAGT	CGCAAAGAAA	TTCCAGGCGA	AAACAGAGAT	2460
GTCTATCTCC	CTCTTTACCA	CACCTGGTAC	CCCTCAAATT	TGAGACCATG	GGACGATCAA	2520
GGAGTCGCTT	TGGGGAATCC	TGTCGAAGGT	GGTAGTGTCA	TCAATTATAC	TGCTAGGATT	2580
GTTGCACCCG	AGGATTATAA	TCTCTTCCAC	AGCGTGGTAC	CAGTCTACGT	TAGAGAGGGT	2640
GCCATCATCC	CGCAAATCGA	AGTACGCCAA	TGGACTGGCC	AGGGGGGAGC	CAACCGCATC	2700
AAGTTCAACA	TCTACCCTGG	AAAGGATAAG	GAGTACTGTA	CCTATCTTGA	TGATGGTGTT	2760
AGCCGTGATA	GTGCGCCGGA	AGACCTCCCA	CAGTACAAAG	AGACCCACGA	ACAGTCGAAG	2820
GTTGAAGGCG	CGGAAATCGC	AAAGCAGATT	GGAAAGAAGA	CGGGTTACAA	CATCTCAGGA	2880
ACCGACCCAG	AAGCAAAGGG	TTATCACC GC	AAAGTTGCTG	TCACACAAAC	GTCAAAAGAC	2940
AAGACGCGTA	CTGTCACTAT	TGAGCCAAAA	CACAATGGAT	ACGACCCTTC	CAAAGAGGTG	3000
GGTGATTATT	ATACCATCAT	TCTTTGGTAC	GCACCAGGTT	TCGATGGCAG	CATCGTCGAT	3060
GTGAGCAAGA	CGACTGTGAA	TGTTGAGGGT	GGGGTGGAGC	ACCAAGTTTA	TAAGAACTCC	3120
GATTTACATA	CGGTTGTTAT	CGACGTGAAG	GAGGTGATCG	GTACCACAAA	GAGCGTCAAG	3180
ATCACATGTA	CTGCCGCTTA	A				3201

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3213 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ATGGCAGGAT	TATCCGACCC	TCTCAATTTT	TGCAAAGCAG	AGGACTACTA	CGCTGCTGCC	60
AAAGGCTGGA	GTGGCCCTCA	GAAGATCATT	CGCTATGACC	AGACCCCTCC	TCAGGGTACA	120
AAAGATCCGA	AAAGCTGGCA	TGCGGTAAAC	CTTCTTTTCG	ATGACGGGAC	TATGTGTGTA	180
GTGCAATTTC	TCAGACCCTG	TGTTTGGAGG	GTTAGATATG	ACCCAGTGT	CAAGACTTCT	240
GATGAGTACG	GCGATGAGAA	TACGAGGACT	ATTGTACAAG	ACTACATGAC	TACTCTGGTT	300
GGAAACTTGG	ACATTTTCAG	AGGTCTTACG	TGGGTTTCTA	CGTTGGAGGA	TTCGGGCGAG	360
TACTACACCT	TCAAGTCCGA	AGTCACTGCC	GTGGACGAAA	CCGAACGGAC	TCGAAACAAG	420
GTGCGCGACG	GCCTCAAGAT	TTACCTATGG	AAAAATCCCT	TTGCGATCCA	GGTAGTGCCT	480
CTCTTGACCC	CCCTGGTGGG	CCCTTTCCCC	ATTCCCAACG	TAGCCAATGC	CACAGCCCGT	540
GTGGCCGACA	AGGTTGTTTT	GCAGACGTCC	CCGAAGACGT	TCAGGAAAAA	CTTGCATCCG	600
CAGCATAAGA	TGTTGAAGGA	TACAGTCTT	GATATTATCA	AGCCGGGGCA	CGGAGAGTAT	660
GTGGGTTGGG	GAGAGATGGG	AGGCATCGAG	TTTATGAAGG	AGCCAACATT	CATGAATTAT	720
TTCAACTTTG	ACAAATATGCA	ATATCAGCAG	GTCTATGCAC	AAGGCGCTCT	TGATAGTCGT	780
GAGCCGTTGT	ATCACTCTGA	TCCCTTCTAT	CTCGACGTGA	ACTCCAACCC	AGAGCACAAG	840
AACATTACGG	CAACCTTTAT	CGATAACTAC	TCTCAGATTG	CCATCGACTT	TGGGAAGACC	900
AACTCAGGCT	ACATCAAGCT	GGGTACCAGG	TATGGCGGTA	TCGATTGTTA	CGGTATCAGC	960
GCGGATACGG	TCCCGGAGAT	TGTGCGACTT	TATACTGGAC	TTGTTGGGCG	TTCGAAGTTG	1020
AAGCCAGGT	ATATTCTCGG	AGCCCAACAA	GCTTGTTATG	GATACCAGCA	GGAAAGTGAC	1080
TTGCATGCTG	TTGTTTCAGCA	GTACCGTGAC	ACCAAGTTTC	CGCTTGATGG	GTTGCATGTC	1140
GATGTGCACT	TTCAGGACAA	TTTCAGAACG	TTTACCACTA	ACCCGATTAC	GTTCCCTAAT	1200
CCCAAAGAAA	TGTTTACCAA	TCTAAGGAAC	AATGGAATCA	AGTGTTCAC	CAACATCACC	1260
CCTGTTATCA	GTATCAGAGA	TCGCCCCAAT	GGGTACAGTA	CCCTCAATGA	GGGATATGAT	1320
AAAAAGTACT	TCATCATGGA	TGACAGATAT	ACCGAGGGGA	CAAGTGGGGA	CCCGCAAAAT	1380
GTTGATACT	CTTTTTACGG	CGGTGGGAAC	CCGGTTGAGG	TTAACCCCTAA	TGATGTTTGG	1440
GCTCGGCCAG	ACTTTGGAGA	CAATTATGAC	TTCCCTACGA	ACTTCAACTG	CAAAGACTAC	1500
CCCTATCATG	GTGGTGTGAG	TTACGGATAT	GGGAATGGCA	CTCCAGGTTA	CTACCCTGAC	1560
CTTAACAGAG	AGGAGGTTCT	TATCTGGTGG	GGATTGCAGT	ACGAGTATCT	CTTCAATATG	1620
GGACTAGAGT	TTGTATGGCA	AGATATGACA	ACCCAGCGCA	TCCATTCATC	ATATGGAGAC	1680
ATGAAAGGGT	TGCCCACCCG	TCTGCTCGTC	ACCGCCGACT	CAGTTACCAA	TGCCTCTGAG	1740
AAAAAGCTCG	CAATTGAAAG	TTGGGCTCTT	TACTCCTACA	ACCTCCATAA	AGCAACCTTC	1800
CACGGTCTTG	GTCGCTTTGA	GTCTCGTAAG	AACAAACGTA	ACTTCATCCT	CGGACGTGGT	1860
AGTTACGCCG	GTGCCTATCG	TTTTGCTGGT	CTCTGGACTG	GAGATAACGC	AAGTACGTGG	1920
GAATTCCTGGA	AGATTTCCGGT	CTCCCAAGTT	CTTTCTCTAG	GTCTCAATGG	TGTGTGTATA	1980

GCGGGGTCTG	ATACGGGTGG	TTTTGAGCCC	GCACGTACTG	AGATTGGGGA	GGAGAAATAT	2040
TGCAGTCCGG	AGCTACTCAT	CAGGTGGTAT	ACTGGATCAT	TCCTTTTGCC	ATGGCTTAGA	2100
AACCACTACG	TCAAGAAGGA	CAGGAAATGG	TTCCAGGAAC	CATACGCGTA	CCCCAAGCAT	2160
CTTGAAACCC	ATCCAGAGCT	CGCAGATCAA	GCATGGCTTT	ACAAATCTGT	TCTAGAAATT	2220
TGCAGATACT	GGGTAGAGCT	AAGATATTCC	CTCATCCAGC	TCCTTTACGA	CTGCATGTTT	2280
CAAAACGTGG	TCGATGGTAT	GCCACTTGCC	AGATCTATGC	TCTTGACCGA	TACTGAGGAT	2340
ACGACCTTCT	TCAATGAGAG	CCAAAAGTTC	CTCGATAACC	AATATATGGC	TGGTGACGAC	2400
ATCCTTGTAG	CACCCATCCT	CCACAGCCGT	AACGAGGTTT	CGGGAGAGAA	CAGAGATGTC	2460
TATCTCCCTC	TATTTCCACAC	CTGGTACCCC	TCAAACCTGA	GACCGTGGGA	CGATCAGGGA	2520
GTCGCTTTAG	GGAATCCTGT	CGAAGGTGGC	AGCGTTATCA	ACTACACTGC	CAGGATTGTT	2580
GCCCCAGAGG	ATTATAATCT	CTTCCACAAC	GTGGTGCCGG	TCTACATCAG	AGAGGGTGCC	2640
ATCATTCCCG	AAATTCAGGT	ACGCCAGTGG	ATTGGCGAAG	GAGGGCCTAA	TCCCATCAAG	2700
TTCAATATCT	ACCCTGGAAA	GGACAAGGAG	TATGTGACGT	ACCTTGATGA	TGGTGTTAGC	2760
CGCGATAGTG	CACCAGATGA	CCTCCCGCAG	TACCGCGAGG	CCTATGAGCA	AGCGAAGGTC	2820
GAAGGCAAAAG	ACGTCCAGAA	GCAACTTGCG	GTCATTCAAG	GGAATAAGAC	TAATGACTTC	2880
TCCGCCTCCG	GGATTGATAA	GGAGGCAAAAG	GGTTATCACC	GCAAAGTTTC	TATCAAACAG	2940
GAGTCAAAAG	ACAAGACCCG	TACTGTCACC	ATTGAGCCAA	AACACAACGG	ATACGACCCC	3000
TCTAAGGAAG	TTGGTAATTA	TTATACCATC	ATTCTTTGGT	ACGCACCGGG	CTTTGACGGC	3060
AGCATCGTCG	ATGTGAGCCA	GGCGACCGTG	AACATCGAGG	GCGGGGTGGA	ATGCGAAATT	3120
TTCAAGAACA	CCGGCTTGCA	TACGGTTGTA	GTCAACGTGA	AAGAGGTGAT	CGGTACCACA	3180
AAGTCCGTCA	AGATCACTTG	CACTACCGCT	TAG			3213

SEQ. ID. NO. 11

SEQUENCE TYPE: NUCLEIC ACID
 MOLECULE TYPE: DNA (GENOMIC)
 ORIGINAL SOURCE: ALGAL
 SEQUENCE LENGTH: 3279 BP
 STRANDEDNESS: DOUBLE
 SEQUENCE:

	10	20	30	40	50	60
1	ATGTTTCTTA	CCCTGACCTT	CATAGCGCCC	AGCGCGCTGG	CCGCCAGCAC	CTTTGTGGGC
61	GCGGATATCC	GATCGGGCAT	TCGCATTCAA	TCCGCTCTTC	CGGCCGTGCG	CAACGCTGTG
121	CGCAGGAGCA	AACATTACAA	TGTATCCATG	ACCGCATTTG	CTGACAAGCA	AACCGCTATC
181	AGTATTGGCC	CTGACAATCC	GGACGGTATC	AACTACCAAA	ACTACGATTA	CATCCCTGTA
241	GCGGGCTTTA	CGCCCTCTC	CAACACCAAC	TGGTATGCTG	CCGGCTCTTC	CACTCCGGGC
301	GGCATCACCG	ACTGGACCGC	TACCATGAAT	GTCAAATTCG	ACCGCATTGA	CAATCCGTCG
361	TACTCCAATA	ACCATCCTGT	TCAGATTTCAG	GTCACGTCGT	ACAACAACAA	CAGCTTCAGG
421	ATTCGCTTCA	ACCCTGATGG	CCCCATTTCG	GACGTCTCTC	GAGGACCTAT	CCTGAAACAG
481	CAACTCACTT	GGATTTCGAA	CCAGGAGCTG	GCGCAGGGAT	GTAATCCGAA	CATGAGCTTC
541	TCTCCTGAAG	GTTTTTTGTC	TTTTGAAACC	AAAGACCTAA	ACGTTATAAT	CTACGGCAAC
601	TGCAAGATGA	GAGTCACGAA	GAAGGATGGC	TACCTCGTCA	TGGAGAATGA	CGAGTGCAAC
661	TCGCAATCAG	ATGGCAATAA	GTGTAGAGGA	TTGATGTACG	TTGACCGGCT	ATACGGTAAT
721	GCTATTGCTT	CCGTACAAAC	GAATTTTCAC	AAAGACACTT	CTCGGAACGA	GAAATTCTAT
781	GGTGCAGGTG	AAGTCAACTG	TCGCTATGAG	GAGCAGGGTA	AGGCGCCGAC	TTATGTTCTA
841	GAACGCTCTG	GACTCGCCAT	GACCAATTAC	AATTACGACA	ACTTTGAACTA	CAACCAACCA
901	GACGTCGTTT	CTCCAGGTTA	TCCCGACCAT	CCCAACTACT	ACATTCCAAT	GTACTACGCA
961	GCACCGTGGT	TGGTCGTTCA	GGGATGCGCG	GGGACATCGA	AGCAATACTC	GTACGGTTGG
1021	TTTATGGACA	ATGTCTCTCA	GTCGTACATG	AACACTGGAG	ATACGGCGTG	GAACGCGGGA
1081	CAGGAAAACC	TGGCATACAT	GGGCGCGCAA	TACGGGCCAT	TTGATCAGCA	CTTTGTGTAT
1141	GGTGATGGAG	ATGGCCTTGA	AGATGTGCTC	AAAGCGTTCT	CCTTTCTTCA	AGGAAAGGAG
1201	TTCGAAGACA	AAAACTCAA	CAAGCGTTCT	GTAATGCCTC	CGAAGTACGT	GTTTGGTTTC
1261	TTCCAGGGTG	TTTTCGGTGC	ACTTTCACTG	TTGAAGCAGA	ATCTGCCTGC	CGGAGAGAAC
1321	AACATCTCAG	TGCAAGAGAT	TGTGGAGGGT	TACCAGGATA	ACGACTACCC	CTTTGAAGGG
1381	CTCGCGGTAG	ATGTTGATAT	GCAAGATGAT	CTCGAGTGT	TTACTACCAA	ACCAGAATAT
1441	TGGTCCGCCAA	ACATGGTAGG	CGAAGGCGGT	GATCCTAATA	ACAGATCAGT	CTTTGAATGG
1501	GCACATGACA	GGGGCCTTGT	CTGTACAGCG	AACGTAACCT	GCTTCTTGAG	GAACGATAAC
1561	AGTGGGAAAC	CATACGAAGT	GAATCAGACA	TTGAGGGAGA	AACAGTTGTA	TACGAAGAAT
1621	GATTCTCTGA	ACAACACCGA	TTTTGGAAT	ACCTCGGATG	GGCCTGGCGA	TGCGTACATT
1681	GGACATTTGG	ACTATGGTGG	TGGAGTGGAG	TGTGATGCAA	TCTTCCCAGA	CTGGGGTCGA
1741	CCAGACGTGG	CTCAATGGTG	GGGAGAAAAC	TACAAGAAGC	TGTTCCAGCAT	TGGTCTCGAT
1801	TTCGTGTGGC	AGGATATGAC	GGTACCTGCG	ATGATGCCGC	ACCGACTCGG	TGATGCTGTC
1861	AACAAAAAAT	CCGGTAGTTC	GGCGCCGGGC	TGGCCGAATG	AGAACGATCC	ATCCAACGGA
1921	CGATACAATT	GGAATCTTTA	TCATCCGCAA	GTGCTCGTGA	CCGACATGCG	CTATGGTGCA
1981	GAGTATGGAA	GGGAACCGAT	GGTGTCTCAA	CGCAACATTC	ACGCCTACAC	TCTTTGTGAA
2041	TCTACCAGAC	GGGAGGGAAT	TGTGGGAAAC	GCAGACAGTT	TGACCAAGTT	CCGCCGCGAGT

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2101 TACATCATCA GTCGAGGAGG TTACATCGGT AACCAGCATT TCGGAGGGAT GTGGGTTGGG
2161 GACAACAGTG CCACAGAATC CTACCTCCAA ATGATGTTGG CGAACATTAT CAACATGAAT
2221 ATGTCGTGCC TCCCCTAGT TGGCTCTGAT ATTGGCGGGT TCACCCAGTA CAATGATGCG
2281 GCGGACCCAA CCCCCGAGGA TTTGATGGTA AGATTTCGTG AGGCTGGCTG TCTGCTACCG
2341 TGGTTCAGAA ACCACTATGA CAGGTGGATT GAGTCCAAGA AGCACGGGAA GAAATACCAG
2401 GAGTTATACA TGTACCCGGG GCAAAAGGAT ACGTTGAAGA AGTTCGTTGA ATTCCGCTAC
2461 CGCTGGCAGG AGGTTTTGTA CACAGCCATG TACCAAAATG CTACCACTGG AGAGCCGATC
2521 ATCAAGGCGG CGCCCATGTA CAACAACGAC GTCAACGTGT ATAAATCGCA GAATGATCAT
2581 TTCCTTCTCG GTGGACATGA CGGCTATCGT ATTCTCTGCG CACCTGTTGT GCGCGAAAAT
2641 GCGACAAGTC GCGAAGTGTA CCTGCCTGTG TATAGCAAGT GGTTCAAATT CGGACCGGAC
2701 TTTGACACTA AGCCCTTGGG AAATGAGATT CAAGGAGGTC AGACGCTTTA TAATTACGCT
2761 GCACCGCTGA ACGATTCGCC GATATTTGTG AGGGAAGGGA CTATTCTTCC GACACGGTAC
2821 ACGCTGGACG GTGTGAACAA ATCTATCAAC ACGTACACAG ACAATGATCC GCTTGATTTT
2881 GAGCTGTTCC CTCTCGAAAA CAACCAGGCG CATGGCTTGT TCTATCATGA TGATGGCGGT
2941 GTCACCAACA ACGCTGAAGA CTTTGCCAAG TATTCTGTGA TCAGTGTGAA GGCCGCGCAG
3001 GAAGGTTCTC AAATGAGTGT CAAGTTTGAC AATGAAGTTT ATGAACACCA ATGGGGAGCA
3061 TCGTTCATAG TTCGTGTTCT TAATATGGGT GCTCCGTCTA ACATCAACGT ATCTTCTCAG
3121 ATTGGTCAAC AGGACATGCA ACAGAGCTCC GTGAGTTCCA GGGCGCAAAT GTTCACTAGT
3181 GCTAACGATG GCGAGTACTG GGTTGACCAG AGCACGAAC TCGTTGTGGCT CAAGTTGCCT
3241 GGTGCAGTTA TCCAAGACGC TGCGATCACT GTTCGTTGA

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SEQ. ID. NO. 12

SEQUENCE TYPE: NUCLEIC ACID
MOLECULE TYPE: DNA (GENOMIC)
ORIGINAL SOURCE: ALGAL
SEQUENCE LENGTH: 1712 BP
STRANDEDNESS: DOUBLE
SEQUENCE:

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          10          20          30          40          50          60
1  ATGACAAACT ATAATTATGA CAATTTGAAC TACAATCAAC CGGACCTCAT CCCACCTGGC
61 CATGATTCAG ATCCTGACTA CTATATTCGG ATGTACTTTG CGGCACCATG GGTGATCGCA
121 CATGGATATC GTGGCACCAG CGACCAGTAC TCTTATGGAT GGTTTTTTGA CAATGTATCC
181 CAGTCCTACA CAAACACTGG CGATGATGCA TGGGCTGGTC AGAAGGATTT GGCCTACATG
241 GGGGCACAAT GTGGGCCTTT CGATCAACAT TTTGTGTATG AGGCTGGAGA TGGACTTGAA
301 GAGGTTGTGA CCGCATTCTC TTATTTGCAA GGCAAGGAAT ATGAGAACCA GGGACTGAAT
361 ATACGTTCTG CAATGCCTCC GAAGTACGTT TTCGGATTTT TCCAAGGCGT ATTCGAGGCC
421 ACATCGCTGC TAAGGGACAA CTTACCTGCC GGCGAGAACA ACGTCTCTTT GGAAGAAATT
481 GTTGAAGGAT ATCAAATCA GAACGTGCCA TTTGAAGGTC TTGCTGTGGA TGTTGATATG
541 CAAGATGACT TGAGAGTGTT CACTACGAGA CCAGCGTTTT GGACGGCAAA CAAGGTGGGG
601 GAAGGCGGTG ATCCAAACAA CAAGTCAGTG TTTGAGTGGG CACATGACAG GGGCCTTGTC
661 TGCCAGACGA ATGTAACCTG CTTCTTGAAG AACGAGAAAA ATCCTTACGA AGTGAATCAG
721 TCATTGAGGG AGAAGCAGTT GTATACGAAG AGTGATTCTT TGGACAACAT TGATTTTGGA
781 ACTACTCCAG ATGGGCCTAG CGATGCGTAC ATTGGACACT TAGACTACGG TGGTGGTGTG
841 GAGTGTGATG CACTATTCCC AGACTGGGGT CGACCAGACG TGGCTCAATG GTGGGGCGAT
901 AACTACAAGA AACTATTTCAG CATTGGTCTC GATTTCTGCT GGCAAGATAT GACGGTACCT
961 GCGATGATGC CGCACCAGCT CGGTGACCCT GTCGGCACAA ATTCGGTGTA GACGGCGCCG
1021 GGCTGGCCGA ATGATAAGGA TCCATCCAAC GGACGATACA ATTGGAAGTC TTACCATCCG
1081 CAAGTGCTCG TGACTGACAT GAGGTATGAC GATTACGGAA GAGATCCCAT TGTTACGCAA
1141 CGCAATCTCC ATGCCTACAC TCTTTGTGAG TCTACTAGGA GGGGAAGGCAT TGTGGAAAC
1201 GCAGATAGTC TGACGAAGTT CCGCCGCAGC TATATTATCA GTCGTGGAGG CTACATCGGT
1261 AATCAGCACT TTGGTGGGAT GTGGGTAGGA GACAACCTCT CTACGGAAGA CTACCTCGCA
1321 ATGATGGTTA TCAACGTTAT CAACATGAAC ATGTCCGGTG TCCCGCTCGT TGGTCCGAT
1381 ATTGGAGGTT TCACGGAGCA TGACAAGAGA AACCTTGCA CACCGGACTT GATGATGAGA
1441 TTTGTGCAGG CTGGATGCTT GCTACCGTGG TTCAGGAACC ACTACGATAG GTGGATCGAG
1501 AGCAAGAAAC ACGGAAAGAA CTACCAAGAG TTGTACATGT ACCCGCACCA CTTGGACGCC
1561 TTGAGAAGTT TTGTGGAAC CCGCTATCGC TGGCAGGAAG TGTTATACAC AGCCATGTAT
1621 CAGAATGCTT TGAACGGGAA GCCGATCATC AAAACGGTCT CCATGTACAA CAACGATATG
1681 AACGTCAAAG ATGCTCAGAA TGACCATTCT CT

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CLAIMS

1. A process of preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the
5 anti-oxidant; and wherein the anti-oxidant is prepared from a glucan by use of recombinant DNA techniques.
2. A process according to claim 1, wherein the glucan comprises α -1,4 links.
- 10 3. A process according to claim 1 or claim 2 wherein the glucan is starch.
4. A process according to any one of the preceding claims wherein the glucan is a substrate for a recombinant enzyme such that contact of the glucan with the recombinant enzyme yields the anti-oxidant.
15
5. A process according to any one of claims 1 to 4, wherein the enzyme is a glucan lyase.
6. A process according to claim 5, wherein the enzyme is an α -1,4-glucan lyase.
20
7. A process according to claim 6, wherein the enzyme comprises any one of the sequences shown as SEQ ID Nos 1-6, or a variant, homologue or fragment thereof.
8. A process according to claim 7, wherein the enzyme is any one of the
25 sequences shown as SEQ ID Nos 1-6.
9. A process according to any one of claims 5 to 8, wherein the enzyme is encoded by a nucleotide sequence comprising any one of the sequences shown as SEQ ID Nos 7-12, or a variant, homologue or fragment thereof.
30
10. A process according to claim 9, wherein the enzyme is encoded by a nucleotide sequence having any one of the sequences shown as SEQ ID Nos 7-12.

11. A process according to any one of the preceding claims, wherein the anti-oxidant is anhydrofructose.
12. A process according to claim 11, wherein the anti-oxidant is 1,5-D-
5 anhydrofructose.
13. A process according to any one of the preceding claims, wherein the medium is, or is used in the preparation of, a foodstuff.
- 10 14. A process according to claim 13, wherein the foodstuff is a beverage.
15. A process according to claim 14, wherein the beverage is an alcoholic beverage.
- 15 16. A process according to claim 14, wherein the beverage is a wine.
17. A process according to any one of the preceding claims, wherein the anti-oxidant is prepared *in situ* in the component and is then released into the medium.
- 20 18. A process according to any one of the preceding claims, wherein the component is a plant or a part thereof.
19. A process according to claim 18, wherein the component is all or part of a cereal or a fruit.
- 25 20. A process according to claim 20, wherein the component is all or part of a grape.
21. A process of preparing a medium that comprises an anti-oxidant and at least
30 one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; and wherein the anti-oxidant is prepared by use of a recombinant glucan lyase.

22. A process according to claim 21 wherein the glucan lyase is that as defined in any one of claims 6 to 10.
23. A medium prepared by the process according to any one of the preceding
5 claims.
24. Use of anhydrofructose as an anti-oxidant for a medium comprising at least one other component, wherein the anhydrofructose is prepared *in situ* in the medium.
- 10 25. Use of anhydrofructose as a means for imparting or improving stress tolerance in a plant, wherein the anhydrofructose is prepared *in situ* in the plant.
26. Use of anhydrofructose as a means for imparting or improving the transformation of a grape, wherein the anhydrofructose is prepared *in situ* in the
15 grape.
27. Use of glucan lyase as a means for imparting or improving stress tolerance in a plant, wherein the glucan lyase is prepared *in situ* in the plant.
- 20 28. Use of glucan lyase as a means for imparting or improving the transformation of a grape, wherein the glucan lyase is prepared *in situ* in the grape.
29. Use of a nucleotide sequence coding for a glucan lyase as a means for imparting or improving stress tolerance in a plant, wherein the nucleotide sequence
25 is expressed *in situ* in the plant.
30. Use of a nucleotide sequence coding for a glucan lyase as a means for imparting or improving the transformation of a grape, wherein the nucleotide sequence is expressed *in situ* in the grape.
30
31. A process or medium substantially as described herein.

ABSTRACT

A PROCESS OF PREPARING AN ANTI-OXIDANT

5 A process of preparing an anti-oxidant is described. The process comprises preparing
a medium that comprises an anti-oxidant and at least one other component. The
process comprises preparing *in situ* in the medium the anti-oxidant. The anti-oxidant
is prepared from either a glucan by use of recombinant DNA techniques and/or by
use of a recombinant glucan lyase.